

I. Preliminary Remarks and Amendments

Claims 1, 4-8, 10, 51-55, 70 and 72 are currently pending and are under examination in the present application. New claim 73 has been added. Support for new claim 73 is found throughout the specification, including at page 112, lines 1-18, wherein the specification recites that agp-96614 messenger RNA was expressed predominantly in human testis cells.

Accordingly, the amendments do not include new matter. Applicants do not intend, with these or any other amendments, to abandon the subject matter of claims previously presented, and reserve the right to pursue such subject matter in duly filed continuing patent applications.

II. Patentability Arguments

A. The Rejection of Claims 1, 4-8, 10, 51-55, 70 and 72 under 35 U.S.C. §101 May Properly Be Withdrawn.

The Examiner rejected claims 1, 4-8, 10, 51-55, 70 and 72 under 35 U.S.C. §101 as assertedly not being supported by either a specific and substantial utility or a well-established utility. Office Action at pages 2-8. In response, Applicants respectfully traverse.

In the Office Action, the Examiner supported the rejection by initially asserting that the identification of the isolated nucleic acid comprising a nucleotide sequence set forth in SEQ ID NO: 1, which is preferentially expressed in testis cells, is not sufficient to impart a specific and substantial biological role for this nucleic acid without further information about the specific properties of this polynucleotide. *See* Office Action at pages 2-3. The Examiner asserted that there is no disclosure for the use of the claimed subject matter as a marker for metastasized testicular cancer cells in the application as filed and this use is not substantial. *Id.* at page 3. Further, the Examiner asserted that specification does not provide a comparative analysis of the expression of the claimed nucleic acid in the pancreas, testis, colon carcinoma cell line (CX-1), and ovarian carcinoma cell line (GI-102). *Id.* at page 4. In addition, the Examiner maintained that the asserted utility of the claimed subject matter, as a marker for testicular cancer cell metastasis, was neither specific nor substantial because any and all nucleic acids expressed in testis tissue can be used in a research setting to detect the presence of testis tissue and differential expression of the

claimed nucleic acid in normal and metastasized testis tissue has not been shown. *Id.* at pages 4-5. The Examiner also took the position that even if the claimed nucleic acid could be used to detect cancerous testicular cells that have metastasized, the utility is not specific because one of skill would not know if the metastasis was due to metastasized testicular or metastasized pancreatic cells because there is a lack of selective expression in testicular cells alone. *Id.* at pages 5-6. The Examiner also contended that because the utility of the claimed nucleic acid, as being up-regulated in testicular cancer cells, is not asserted in the specification, and because it is not specific, one of skill would have to conduct further experiments to determine the role of the claimed nucleic acid in the pancreas and testis. Because it is assertedly not clear whether the claimed nucleic acid is up-regulated or down-regulated in cancer, the skilled artisan would not know if it were desirable to identify drugs that antagonize or agonize the protein encoded by the claimed nucleic acid. *Id.* at pages 7-8. The Examiner concluded by stating that the credibility of the claimed nucleic acid has never been questioned; however, the specification failed to provide a specific and substantial, or well-established utility for the claimed nucleic acid. *Id.* at page 8. Applicants address each of the Examiner's arguments in the following remarks.

To satisfy the requirements of 35 U.S.C. §101, an applicant must claim an invention that is statutory subject matter and must show that the claimed invention is "useful" for some purpose either explicitly or **implicitly**." M.P.E.P. §2107.01 (emphasis added). Furthermore, an invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., **properties** or applications of a product or process), and (ii) the utility is specific, substantial, and credible. M.P.E.P. §2107.01 (emphasis added). If an invention has a well-established utility, rejections under 35 U.S.C. §101 and 35 U.S.C. §112, first paragraph, based on lack of utility should not be imposed. *In re Folkers*, 344 F.2d 970 (CCPA 1965). M.P.E.P §2107.02 further provides an example that if an application teaches the cloning and characterization of the nucleotide sequence of a well-known protein, such as insulin, and those skilled in the art at the time of filing knew that insulin had a well-established use, it would be improper to reject the claimed invention as lacking utility solely because of the omitted statement of specific and substantial utility.

In support of that utility, the application-as-filed provided experimental evidence establishing an elevated level of expression of a novel human gene, agp-96614-a1, (CD20/IgE receptor-like mRNA; SEQ ID NO: 1) in the human testis after performing Northern blot and RT-PCR analyses. *See* specification, Example 3 at page 112. The present application provides an alignment of the amino acid sequence of the polypeptide (SEQ ID NO: 2) encoded by this gene with other members in the CD20 family (*see* Figure 3). Thus, one of skill in the art would recognize that Applicants identified a novel testis-specific form of a CD20/IgE receptor-like molecule. The agp-96614-a1 protein is shown to be 200 amino acids, and other members of this family of proteins have been shown to contain four putative transmembrane regions, N- and C-terminal cytoplasmic domains, and three inter-transmembrane loop regions. In fact, three later publications identified the exact same molecule (i.e., agp-96614-a1) as being a testis-expressed transmembrane 4 protein (Ishibashi et al., *Gene* 264:87-93, 2001; Liang et al., *Genomics* 72:119-127, 2001; Hulett et al., *Biochem. Biophys. Res. Commun.* 280:374-379, 2001; *see* Appendix C of the response filed January 15, 2003). Thus, the application disclosed the complete structures of a protein and encoding nucleic acid and showed preferentially elevated expression in testis cells.

In response to the Examiner's assertion that the property of the isolated nucleic acid (SEQ ID NO: 1) of being preferentially expressed in testis cells is not sufficient to impart a specific and substantial biological role for this nucleic acid without further information about the specific properties of this polynucleotide, Applicants respectfully disagree. A person having ordinary skill in the art will recognize a specific and substantial biological role for a novel testis-specific protein. As Applicants have previously established, a testis-specific protein is useful as a tissue-specific marker for detecting testis cells which have moved outside of the testis, either as a micrometastatic deposit of cells into the lymph nodes or bone marrow, or as a secretion into the bloodstream to indicate the presence of an abnormal condition of the testis, such as metastasized testicular cancer. Tissue-specific markers are not specifically related to cancer necessarily, but elevated levels of a tissue-specific mRNA or protein are useful for identifying a specific tissue as being at fault or suspect (*see* Exhibit A; "tissue-specific markers" as defined by Wikipedia, an online encyclopedia). For example, prostate-specific antigen (PSA) is a protein which is preferentially expressed in the prostate and has proven to be a very useful human tissue-specific marker for detecting prostate abnormalities, either as an indicator of prostate cells

which have moved outside of the prostate as a tumor (i.e., metastatic prostate cancer) or as an elevated level of PSA in the bloodstream, which can indicate the presence of prostate cancer or benign prostatic hyperplasia (Mulders et al., *Eur. J. Surg. Oncol.* 16:37-41, 1990; see Exhibit B).

Moreover, a person having ordinary skill in the art will recognize that genes that are expressed in a highly tissue-specific manner are targets for therapeutics, early detection of cancer, and monitoring of disease burden during and after treatment (Brown et al., *Breast Cancer Res. Treat.* Dec. 1:1-7, 2005; see Exhibit C). For example, mammaglobin mRNA and protein, found in breast epithelial cells, are of high interest as diagnostic markers for breast cancer (Zehenter et al., *Clin. Biochem.* 37:249-257, 2004; see Exhibit D).

Further, in response to the Examiner's assertion that there is no disclosure for the use of the claimed subject matter as a marker for metastasized testicular cancer cells in the application as filed and that this use is not substantial, Applicants respectfully disagree. At minimum, there is an implicit disclosure of the use of the claimed subject matter as a marker to testicular cell dissemination or metastasis, and such a disclosure is sufficient under 35 U.S.C. §101. As stated in M.P.E.P. §2107.03, "a reasonable correlation between the evidence and the asserted utility is sufficient to prove utility." Applicants submit that they have established this reasonable correlation (see, e.g., articles in scientific journals made of record and argument provided herein and in previous responses of record).

In response to the Examiner's assertion that the specification does not provide a comparative analysis of the expression of the claimed nucleic acid in the pancreas, testis, colon carcinoma cell line (CX-1), and ovarian carcinoma cell line (GI-102), Applicants again respectfully disagree. The specification states that PCR analysis detected agp-96614-a1 in human testes, pancreas, a colon adenocarcinoma cell line (CX-1), and an ovarian carcinoma cell line (GI-102); however, "Northern blot analysis of the MTE blots (Clontech, CA) indicated that agp-96614-a1 was expressed predominantly in human testis." See specification at page 112, lines 13-17. Thus, a comparative analysis of the expression of the claimed nucleic acid was summarized in the specification, and drawings (e.g., a Northern blot) are not required in a patent application if they are not necessary for an understanding of the subject matter of the invention. 37 C.F.R. §1.81. Moreover, the multiple tissue expression (MTE)

blots (Clontech) used in the gene expression experiments described in the specification (*see* pages 112-114), contained 76 poly A⁺ RNAs (*see* Exhibit E) and provided a virtual “whole body image” of gene expression in all tissues of the body. Having disclosed that such Northern blots were performed and having disclosed that the results showed predominant expression in the testis, the application provided sufficient disclosure to support the utility of the claimed subject matter notwithstanding the Examiner's preference for a different form for disclosing those results.

Further, in response to the Examiner's assertion that the utility of the claimed subject matter, as a marker for testicular cancer cell metastasis, was neither specific nor substantial because any and all nucleic acids expressed in testis tissue can be used in a research setting to detect the presence of testis tissue and differential expression of the claimed nucleic acid in normal and metastasized testis tissue has not been shown, Applicants respectfully submit that differential expression does not need to be shown to establish the utility of agp-96614-a1 as a testis-specific marker. As set out above, a tissue-specific marker is useful in simply signaling that the testicular cell or protein is being disseminated outside of the testis. The marker's usefulness is not solely based on differential expression. In fact, Applicants' assertion of utility of agp-96614-a1 as a marker for disseminated testicular cells is not dependent on a differential level of expression of agp-96614-a1 in cancerous *versus* healthy cells of the testis. The marker, agp-96614-a1, is useful for its ability to identify a testicular cell. Identification of testicular cells in a body location other than in the testis would be recognized by one of ordinary skill in the art as the identification of a cancerous testicular cell that had metastasized. Accordingly, the asserted utility does not require a detectable difference in expression in cancerous *versus* healthy testicular cells and there is, therefore, no need for comparative studies of cancerous and healthy testicular cells to support that utility.

The Examiner also took the position that even if the claimed nucleic acid could be used to detect cancerous testicular cells that have metastasized, the utility is not specific because one of skill would not know if the metastasis was due to metastasized testicular or metastasized pancreatic cells because there is a lack of selective expression in testicular cells alone. In response, Applicants yet again respectfully disagree and reiterate their position that the predominant expression of the claimed nucleic acid in human testis

provides a distinguishably detectable level of expression in testis regardless of any expression in pancreas or carcinoma cell lines. The expression in pancreas and carcinoma cell lines, however, was determined using the sensitive technique of PCR. In contrast, the statement in the application that expression was predominantly found in human testis was based on Northern blot data. The two statements regarding expression are not inconsistent – there is a detectable level of expression in pancreas (and in two *ex vivo* cell lines), but the predominant expression is found in human testis. Thus, the Examiner's reliance on expression in pancreas and two cell lines to undermine the express disclosure of a predominant expression in human testis is misplaced. Further, the subsequent publications of Ishibashi et al., *Gene* 264:87-93, 2001; Liang et al., *Genomics* 72:119-127, 2001; and Hulett et al., *Biochem. Biophys. Res. Commun.* 280:374-379, 2001 (see Appendix C of the response mailed January 15, 2003) support Applicants' position that this protein is a testis-specific protein. Moreover, PSA immunoreactivity has been reported in seminal vesicle epithelium and in non-prostatic tumors of the salivary gland, breast, kidney, bladder, colon, and lung (Varma et al., *Am. J. Clin. Pathol.* 118: 202-207, 2002; see Exhibit F); however, it has not eliminated the utility of PSA as a significantly useful prostate-specific marker in human medicine. Consequently, the claimed utility of agp-96614-a1, as a testis-specific marker, would be recognized as specific by one of skill in the art.

Last, the Examiner stated that because the utility of the claimed nucleic acid, as being up-regulated in testicular cancer, is not asserted in the specification, and because it is not specific, one of skill would have to conduct further experiments to determine the role of the claimed nucleic acid in the pancreas and testis. The Examiner further asserted that it is not clear whether the claimed nucleic acid is up-regulated or down-regulated in cancer. Thus, the Examiner asserted that a skilled artisan would not know if it were desirable to identify drugs that antagonize or agonize the protein encoded by the claimed nucleic acid. Applicants respectfully submit that this argument is irrelevant to the issue of satisfying the requirements of 35 U.S.C. §101. Regardless of whether the claimed nucleic acid is up-regulated or down-regulated in testicular cancer, or expressed at the same level as in healthy cells of the testis, Applicants have simply maintained that the claimed nucleic acid is a testis-specific gene and, because it is a testis-specific gene, it is useful as a marker for a testicular cell or a testis-specific protein that has spread to other parts of the body (i.e., spread beyond the testis).

Because the Examiner has stated that the credibility of the claimed nucleic acid has never been questioned and because Applicants have shown that the claimed nucleic acid has a specific and substantial utility, or a well-established utility, Applicants submit that they have provided evidence of an asserted diagnostic use of the claimed compound by providing a reasonable correlation between the activity in question, i.e., that a polypeptide having the amino acid sequence set forth in SEQ ID NO: 2 is expressed at an elevated level in testicular tissue as compared to other tissues. As previously presented, there is no data contradicting the data showing elevated expression in the testis. In view of the data disclosed in the application, not only standing unopposed but being corroborated by subsequent disclosures (*see* Appendix C of the response mailed January 15, 2003), there is no question that one of skill would take a position consistent with the only data available and be less likely, not more likely, than not to doubt the asserted utility. The subsequent identification of the exact same molecule (sharing 100% identity) by three independent authors, as being a testis-expressed transmembrane 4 protein, affirms Applicants' position that the claimed molecule is useful in identifying testicular cells, especially in identifying the metastasis of testicular cancer.

For all of the foregoing reasons, the Examiner has not established that the use of the claimed subject matter as a marker for testicular cells, especially for metastasized testicular cancer cells, is not specific or substantial. As already noted, the Examiner has not challenged the credibility of any assertions. Thus, the specification provides a credible, substantial, and specific, or well-established, asserted utility for the invention as claimed. Accordingly, the rejection of all pending claims under 35 U.S.C. §101 for an asserted lack of utility should be withdrawn.

B. The Rejection of Claims 1, 4-8, 10, 51-55, 70 and 72 under 35 U.S.C. §112, First Paragraph, May Properly Be Withdrawn.

The Examiner also rejected claims 1, 4-8, 10, 51-55, 70 and 72 under 35 U.S.C. §112, first paragraph, for lack of enablement based on the asserted lack of utility. *See* Office Action at page 8. The basis for the rejection is defective in relying on an asserted lack of patentable utility, as established above. For that reason, the rejection of claims 1, 4-8, 10,

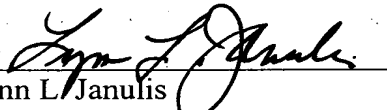
51-55, 70 and 72 under 35 U.S.C. §112, first paragraph, for lack of enablement, has been overcome and should be withdrawn.

CONCLUSION

In view of the preceding remarks, Applicants submit that claims 1, 4-8, 10, 51-55, 70, 72, and 73 are in condition for allowance. Expedited notification thereof is respectfully requested.

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Respectfully submitted,

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Tumor marker

From Wikipedia, the free encyclopedia

Tumor markers are substances found in the blood, urine or body tissues that can be elevated in cancer. There are many different tumor markers. They are used in oncology to help determine the presence of cancer. An elevated level of a tumor marker can indicate cancer, however there can often also be other causes of the elevation.

Tumor markers can be produced directly by the tumor or by non-tumor cells as a response to the presence of a tumor.

Tumor markers can be used in screening programs, such as screening for elevated levels of prostate specific antigen to indicate possible prostate cancer. Tumor markers are also used to monitor patients for cancer return.

Tumor markers can be classified in two groups: Cancer-specific markers and tissue-specific markers.

Cancer-specific markers

Cancer-specific markers are related to the presence of cancerous tissue. Because there is a large overlap between the many different tumor types and the markers produced these markers tend to be unhelpful in making a diagnosis. They can, however, be useful in follow up of treated patients to describe progress of the disease before any further masses can be found clinically or by imaging. A few examples of these markers are CEA, CA19-9, CA125.

An example of a cancer-specific marker, CEA, or carcinoembryonic antigen, is a blood-borne protein, first noted to be produced by tumors of the gastrointestinal system. Further investigation showed that it was produced by the occasional lung and breast cancer case, meaning that an elevated level does not mean a bowel cancer. However, in a patient with a history of a treated bowel cancer, a rising CEA level is an early sign of bowel cancer return. This usually occurs BEFORE the site of return can be identified on imaging or examination, and so many oncologists question the wisdom of doing a blood test for CEA when the end result is bad news that terrifies the patient, but does not have much impact on treatment.

Tissue-specific markers

Tissue-specific markers are related to specific tissues which have developed cancer. Generally speaking, these substances are not specifically related to the tumor, and may be present at elevated levels when no cancer is present. But unlike the previous group, elevated levels point to a specific tissue being at fault. Examples include PSA, beta-HCG - (Human chorionic gonadotropin), AFP - (Alpha-fetoprotein), AFP-L3 - (a lectin-reactive AFP) and Thyroglobulin. For example, if man has an elevated PSA, a search for prostate cancer will be undertaken. If an individual has an elevated level of beta-HCG, AFP or AFP-L3%, a search for a testicular or liver cancer, respectively, will be made.

An example of a tissue-specific marker is PSA, or Prostate specific antigen, which is produced by the normal prostate. It is a protein enzyme called a serine protease that usually acts as an anticoagulant to keep semen liquid. Only small amounts leak into the circulation in normal circumstances. Enlarged prostates leak more substantial amounts, and cancerous prostates also leak substantial amounts. An accurate way to tell if an elevated PSA level results from cancer is to biopsy the prostate.

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Categories: Oncology | Tumor markers

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Prostate-specific antigen (PSA). A tissue-specific and sensitive tumor marker

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Noninvasive methods for the diagnosis of prostatic cancer, its staging and evaluation of response to therapy are often not sufficiently sensitive or specific. Prostate-specific antigen (PSA) was identified in 1979 and has been evaluated since then as a marker, both at the serum and the tissue level. A review is presented in this article. PSA is an organ-specific glycoprotein presented in most prostatic carcinomas, but also in normal prostatic tissue and in benign prostatic hypertrophy (BPH). The monitoring of serum PSA concentrations by serial measurement can be used for the detection of residual or recurrent tumor after primary treatment and for the evaluation of response to systemic treatment of advanced disease. At the tissue level immuno-histochemical detection of PSA may help to identify metastatic tumor of unknown origin. PSA serum assays have not been sufficiently sensitive and specific for staging of the primary tumor or for screening purposes. PSA is an equally specific, but more sensitive marker of prostatic carcinoma compared to prostatic acid phosphatase.

Key words: prostate-specific antigen; tumor marker; prostate cancer; benign prostatic hypertrophy.

Introduction

Prostate cancer is the second most common cancer in men over 60 years old in the Western industrialized world. Unfortunately many cases of prostate cancer are only discovered when the neoplasm is no longer confined to the prostate. Therefore, a variety of methods have been studied, including several tumor markers, such as prostatic acid phosphatase (PAP), for their usefulness in the diagnosis and staging of prostate cancer and in the evaluation of response to therapy. None of them, however, seems to be sensitive or specific enough.

In 1979 Wang *et al.*¹ identified prostate-specific antigen (PSA). PSA is a glycoprotein with a molecular weight of 34,000. It is different from prostatic acid phosphatase (PAP) both immunologically and biochemically. PSA is found in normal prostate, benign prostatic hypertrophy (BPH) and in

malignant prostatic tissues, but not in other human tissues. Consequently, PSA is not tumor-specific but it is organ-specific.

The localization of PSA at the ultrastructural level was studied using a protein A-gold complex.^{2,3} These studies showed that PSA was primarily localized in the cytoplasmic vesicles, vacuoles and secretory granules, rough endoplasmic reticulum (RER) and within the glandular lumina of the columnar or cuboidal cells, but not in the basal epithelial cells. PSA is probably synthesized in the RER, stored in vesicles and vacuoles and released into the glandular lumina by exocytosis. This PSA localization was found in normal, BPH and malignant prostatic tissues. In the case of BPH and malignant prostatic tissue, however, PSA was also found in neutrophils and macrophages. In undifferentiated prostatic cancer PSA has also been found in association with membranous structures, probably because specific cytoplasmic organelles in undifferentiated neoplastic cells are not differentiated.²

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The characteristics of PSA as a serum marker have been studied, which will be reviewed in this article.

PSA concentrations in serum

Serum PSA levels can be measured by radioimmunoassay. Both the Tandem-R PSA (Hybritech) assay, a monoclonal solid-phase immunoradiometric assay, and the Pros-Check PSA (Yang) assay, a polyclonal competitive radioimmunoassay, have been widely used. Results of these two assays show close linear correlation,⁴ but the Pros-Check assay yields values 1.5–1.85 times those of the Tandem-R assay.^{4,5} In general, Tandem-R serum PSA levels are less than 4 ng/ml in 99%, less than 10 ng/ml in 100% of apparently healthy men. PSA is found below 4 ng/ml in 100% of men under the age of 40 years, whereas in healthy men over 40, 3% of the serum values range between 4 and 10 ng/ml. Normal ranges may, however, be different in different laboratories. The Tandem-R assay achieves better precision at all concentrations, probably because it is performed with monoclonal antibodies.

For PSA (and PAP), spontaneous 24-h variations of serum values were observed. The variations were greatest in patients with stage D prostatic cancer, who most often showed elevated basal values. On average, spontaneous variability of PSA (as evaluated by coefficient of variation) was about 20% greater compared to PAP, although the fluctuations of both markers in the individual patient were unpredictable and never coincidental.⁶ Schiffman *et al.*,⁷ however, found low intra-individual variance and small fluctuations in serum from day to day compared to PAP and acid phosphatase. These conflicting results could be caused by differences in the assay methods employed. Mannini *et al.*⁶ used a liquid-phase radioimmunoassay (PSA-double antibody, Diagnostic Products Company) and Schiffman *et al.*⁷ a solid-phase two-site radioimmunometric assay (Hybritech, Inc. San Diego, CA). Increases of PSA serum values are caused by manipulations of the prostate, prostate massage (1.5 to 2 times), needle-core perineal biopsy and transurethral resection (50 to 60 times).^{8,9} Such procedures should therefore be avoided prior to blood sampling for measurement of serum PSA. The average serum half-life of PSA was found to be 2.2 to 3.15 days.^{8,10} As a direct implication of this, a serum PSA measurement would be valid only 2 to 3 weeks

after a needle core biopsy or a transurethral resection.

Pre-operative levels of PSA have not been sufficiently reliable to predict the pathological stage of early prostatic carcinoma on an individual basis.¹⁰ According to some authors, PSA levels reflect neither the histological grade nor the local stage of the tumor and are of no value estimating tumor burden.^{11,12} Stamey *et al.*,⁸ however, found that the PSA level increased with advancing clinical stage and was proportional to the estimated volume of the untreated prostate tumor. Ecrole *et al.*¹³ also concluded that PSA is an excellent tumor marker for monitoring patients with prostatic carcinoma and that PSA might even be useful to stage apparently localized carcinoma of the prostate. As stated above, these different observations could also result from the difference in assays used. Oesterling *et al.*,¹⁰ Ferro *et al.*¹¹ and Emtage *et al.*¹² used the Tandem-R PSA (Hybritech) assay, whereas Ecrole *et al.*¹³ and Stamey *et al.*⁸ used the Pros-Check PSA (Yang) assay. Another possible explanation for the difference in results could be that the study of Ecrole *et al.*¹³ is retrospective, whereas the other observations were made prospectively. Because of the elevation of serum PSA levels in some patients with benign prostatic hyperplasia (BPH), PSA has been judged to be of no or little screening value in the detection of prostate cancer.^{5,8,11,13,14,16} At a decision value of 8.0 ng/ml Tandem-R PSA, advanced stages of prostatic cancer could be distinguished from benign prostatic hypertrophy with 90% specificity.⁵

The achievements of PSA as a tumor marker have become evident in the detection of residual disease after primary treatment (radical prostatectomy, cryosurgery or radiation therapy), recurrence of tumor during long-term follow-up and the monitoring of a response to palliative therapy.^{5,8,10,11,13–16} Serum PSA falls to undetectable levels after radical prostatectomy. It is generally found that high PSA levels are strongly suggestive of disease recurrence. Levels of PSA may even be elevated before a recurrence of disease becomes clinically manifest. Killian *et al.*¹⁵ found an inverse relationship between the time-lapse preceding a clinically detectable recurrence of disease and a serum level of PSA. They also found that the mean lead time (the interval between the first of repeatedly elevated PSA levels, or a single elevated PSA level more than 5 times the upper normal limit and a confirmed disease recurrence) was 12 months on average. PSA serum levels decrease when a favourable therapeutic response is achieved.

Comparison with other markers in serum and in tumor tissue

When the clinical usefulness of PSA as a marker of prostatic carcinoma is compared to PAP, PSA is generally found to be more sensitive.⁷⁻¹⁶ Just like PSA, PAP is not tumor-specific, as PAP levels may also be elevated in BPH patients. PSA is also a more sensitive serum tumor marker when compared to acid phosphatase and alkaline phosphatase.

Purnell *et al.*¹⁷ have studied PSA in comparison with carcinoembryonic antigen (CEA), nonspecific cross-reacting antigen (NCA) and β -chorionic gonadotrophin (HCG) in primary prostatic cancer at the tissue level with a peroxidase-antiperoxidase technique. They found that PSA was the most sensitive since it was present in all carcinomas, whereas the other markers appeared to be expressed more frequently in well-differentiated compared to less-differentiated tumors.

It has been suggested that primary prostatic carcinomas are composed of heterogeneous subpopulations of neoplastic cells and that only specific subpopulations seem to have metastatic potential. Fan *et al.*¹⁸ have, therefore, evaluated the p21 protein (a ras oncogene product) as a clinical predictor for metastatic spread. They showed that the expression of p21 differed for the various subpopulations of cells, in contrast to PSA and PAP, which were expressed by almost all cells (85%) in the primary tumor. They also found that almost all of the metastatic tumor cells in skeletal lesions were positive for the expression of p21, whereas about 85% of the metastatic tumor cells were positive for PSA and PAP. It thus seems unlikely that quantification of PSA and PAP in the primary tumor cells will have predictive value for their metastatic potential. Fan *et al.* concluded that the basic limitations of PSA and PAP lie in the fact that both are normal prostatic protein components and therefore not specific as tumor markers. The p21 protein is, however, tumor-specific as it is the abnormal expression of an oncogene.

Comparison of serum PSA with clinical parameters of prostate cancer

Hetherington *et al.*¹⁹ compared changes of PSA and PAP with changes in serial bone scans in patients with prostatic carcinoma. They found that serial measurements can provide a guide to disease activity including both response and progression, although repeated scans could show early progression in the skeleton when PSA and PAP were

still normal. The obvious disadvantage of bone scanning is that it gives no information about the local tumor extension and spread to soft tissues. Furthermore, bone scans are expensive compared to measurements of serum markers.

For the detection of prostatic carcinoma rectal examination and transrectal ultrasonography are widely used. Unfortunately, like the determination of serum PSA and PAP, all have proved imperfect because of insufficient specificity and sensitivity.²⁰

Immunohistochemistry

Morote *et al.*²¹ evaluated whether in cases of prostatic cancer the quantification of bone marrow PSA and PAP can provide more information than the determination of PSA and PAP in serum. They found a significant correlation between serum and bone marrow levels which was independent of the presence of bone metastases. Morote *et al.* thus concluded that bone marrow measurements do not give additional information.

Staining tissue sections for PSA with the immunoperoxidase technique has been demonstrated to be useful for the identification of metastatic prostate cancer (e.g. in cases of unsuspected extra-prostatic spread of the prostate carcinoma), due to the tissue-specificity of PSA.^{22, 23} Goldfarb *et al.*²⁴ used the immunoperoxidase technique to show that tissue PSA (and PAP) levels are age-dependent in a way which could be best explained by a relationship with serum testosterone concentrations.

The value of immunohistological staining for PSA in less differentiated prostatic carcinoma, however, seems questionable.^{17, 25} Staining for PAP appears to be preferable since it is more intense and, therefore, more visible, but also because it is often positive in areas where PSA staining is equivocal or negative.

Immunoperoxidase staining of PSA in decalcified bone marrow biopsies containing metastatic tumor has yielded, except in poorly differentiated prostatic carcinoma, good results.²⁶ This has been explained as an effect of the decalcification procedure rather than an inherent difference in the expression of PAP and PSA in the bone marrow metastases.

Mansi *et al.*²⁷ used a mixture of antisera to PAP, PSA, epithelial membrane antigen (EMA) and cytokeratin to examine multiple bone marrow aspirates from patients with local or metastatic prostatic carcinoma and BPH. They found many positive-stained cells in the bone marrow of 73% of the patients with metastatic prostate cancer, a

small number of cells in 13% of the local cancer patients and no positive cells in any of the BPH patients. Mansi *et al.* suggested that bone marrow immunocytochemistry may increase the detection rate of metastasis.

Conclusions

Measurements of PSA concentrations in serum have proven to be useful for the detection of recurrent disease after primary radical treatment, and the monitoring of a response to palliative therapy of prostatic carcinoma. The PSA assay can predict the clinical recurrence of tumor during long-term follow-up with a mean lead-time of 12 months.

The usefulness of serum PSA assays for the staging of prostate cancer at first diagnosis and for the assessment of prognosis is doubtful, since it has not been shown to be specific and sensitive enough. Serum measurements of PSA are also of little value for screening purposes, because elevated levels may be found in patients with BPH and prostatitis. In addition to BPH and prostatitis, elevated serum PSA concentrations can be caused by manipulation of the prostate, which should therefore be avoided when measurements of serum PSA are intended. Whether impaired liver or kidney function or urinary retention also have an effect on serum PSA concentrations has not been reported yet.

PSA immunohistochemistry facilitates the identification of metastatic cancer of unknown origin. As a result of the tissue-specificity of PSA, the latter is a rare characteristic, as there are only very few known tissue-specific markers. Since PSA is a normal prostate protein component, it is not surprising that PSA was not predictive for the eventual behavior of a carcinoma. Quantification of the ras oncogen p21, however, is more likely to be useful.

Comparison of PSA with other markers in serum, such as PAP and acid phosphatase, determined either immunohistochemically or enzymatically, has demonstrated that PSA is more sensitive. Changes in serial PSA concentrations are often more pronounced compared to PAP or other markers. In contrast, PAP is performing better in immunohistochemistry of less-differentiated prostatic carcinoma.

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Evaluation of expression based markers for the detection of breast cancer cells

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Summary

Introduction. Genes that are expressed in a highly tissue- or disease-specific manner provide possible targets for therapeutics, early detection of cancer, and monitoring of disease burden during and after treatment. Further, genes of this type that code for secreted or shed proteins may allow for serum detection of the product facilitating our ability to specifically detect the cancer in all circumstances. To this end, we are working towards identification and characterization of such genes that are specifically expressed in breast epithelium. In the current study, we have measured the expression of two markers that emerged from a screen of the Incyte LifeSeq Database and were subsequently shown to be highly restricted to breast epithelium termed BU101 (also called Lipophilin B) and BS106 (small mucin-like protein). These two novel markers were compared with two other candidate markers, Mammaglobin and Cytokeratin 19 (CK19).

Methods. Utilizing quantitative real-time PCR, we compared the expression of these four genes in a series of 95 primary breast cancers, 9 lymph nodes from breast cancer patients, 13 lymph nodes from non-cancer patients and 10 normal breast tissues.

Results. Cytokeratin was shown to be highly sensitive in detecting all breast cancers, while BU101, BS106 and Mammaglobin were more restricted.

Conclusion. While no one of these markers efficiently detects all breast cancers, a combination of two or more could achieve a very high sensitivity in assaying for circulating or occult breast cancer cells.

Introduction

Reliable molecular detection of breast cancer cells can be usefully applied in a number of clinical settings including screening, staging, monitoring response to therapy, and detection of recurrent disease. Immunohistochemistry is now commonly used to detect micrometastatic deposits in axillary lymph nodes and bone marrow, however these assays typically rely on antigens expressed by all epithelial cells, i.e., low molecular weight cytokeratins (CK). The presence of CK expressing cells in axillary lymph nodes, bone marrow, and in peripheral blood has been used as a marker for micrometastatic or occult disease [1–5]. Since CK is epithelial but not breast specific, the identity of these rare cells can be open to speculation.

Genes that are expressed exclusively or predominantly by all breast cancers could help address this issue. The search for these genes has been accelerated by the

availability of large expression databases. *In silico* analysis of the Incyte LifeSeq Database produced two new sequences with expression patterns predominantly restricted to breast tissue [6]. The first of these genes, designated in our work as BU101, is a member of the uteroglobin family (also described as Lipophilin B and Secretoglobin family 1D member 2 (SCGB1D2), Hs.204096 [7]). The uteroglobins are part of the secretoglobulin superfamily. They are small secreted proteins that may be involved in signaling, the immune response, and chemotaxis [8–13]. Another member of this family, Mammaglobin A (MGB1), also demonstrates mammary tissue restricted expression and has been studied as a possible breast cancer marker [14]. We have shown that BU101 is co-expressed with mammaglobin in a series of 20 breast specimens and forms heterotetrameric complexes with MGB1 that are secreted from breast cells. The significance of this interaction is not known, however it appears to be the predominant form of both proteins in these cells [6].

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The second cDNA sequence that was largely restricted to the breast and salivary gland was a novel gene with mucin-like properties [15]. This gene, termed BS106, has an open reading frame of 90 amino acids (10.7 kDa predicted M_w) but has an apparent monomeric mobility of approximately 40 kDa consistent with extensive glycosylation characteristic of mucins. Several larger species (80 and > 200 kDa) have been isolated by affinity purification from breast milk indicating the existence of multimeric complexes. This gene was independently identified from another *in silico* screen also searching for breast specific sequences and termed small breast epithelial mucin (Hs.348419) [16].

We have been examining these expression markers for their potential utility in detecting occult breast cancer. The ideal marker would be expressed at high levels by most or all breast cancers, continue to be expressed by metastatic disease, and have low or undetectable expression in all other cells, particularly lymphocytes. In this current study, we have measured the expression of the two new markers, BU101 and BS106 in comparison to the established markers, MGB1 and a specific low molecular cytokeratin, CK19, in a series of primary breast cancers, metastatic lymph nodes and normal lymph nodes by quantitative RT-PCR. CK19 was expressed by all cancer containing specimens, however none of the breast specific markers were detectable in every cancer by this sensitive method. Potentially, a nested approach using the non-specific but sensitive CK19 followed by one or more of the breast specific markers may provide sufficient discrimination to detect and measure occult breast cancer.

Materials and methods:

Tissues specimens and cells

Frozen breast cancer specimens and axillary lymph nodes were obtained from women undergoing cytoreductive surgery for the disease under a Duke IRB approved protocol. This material was only taken after the pathologist determined that sufficient tissue was available for definitive diagnosis. Lymph nodes were obtained from individuals undergoing surgical treatment for non-malignant disease. Only breast cancers with ductal or lobular histology were used in this study. These specimens were banked with the consent of the patient under an IRB approved protocol and maintained at -130°C . SKBR3 cells were obtained from the ATCC and maintained in RPMI1640 containing 10% FBS. The SKBR3 line was used as a positive control and for quantitative normalization since it had abundant expression of all the markers under consideration.

Normal lymphocytes from women with no evidence of malignancy were obtained under an IRB approved protocol. Blood was collected in CPT (cell processing tubes, Becton Dickinson) tubes and buffy coat cells were obtained according to the standard protocols developed

for these tubes. Cells were frozen at -135°C until RNA was extracted as described below.

Information regarding stage, histologic type, and steroid hormone receptor content of the cancer was obtained from pathology records under an IRB approved protocol. HER2/neu protein content was evaluated by immunohistochemical staining using the monoclonal antibody, TA-1 (as previously described [17]). TP53 protein accumulation, a hallmark of missense mutations in this tumor suppressor gene, was assessed by immunohistochemical staining with the monoclonal antibodies DO-1 and 1801 and evaluated as previously described [18,19]. For p53, nuclear accumulation in greater than 10% of the malignant cells was considered positive. For HER2/neu, membrane staining was assessed on a continuous scale based upon intensity. Staining was typically homogeneous throughout the malignant epithelium [17].

RNA extraction

All tissues and blood were obtained under Duke University Medical Center IRB authorization. All tissues were flash frozen within 60 min of devascularization. Before extraction, each tissue specimen was sectioned and stained with hematoxylin and eosin to evaluate epithelial content. For breast cancer specimens, only tissues that exhibited at least 50% malignant epithelium (on a per cell basis) were selected for this study. Approximately 30 mg of tissue was added to a chilled BioPulverizer H tube (Bio101). Lysis buffer from the Qiagen RNeasy Mini kit was added and the tissue was homogenized for 20 s in a Mini-Beadbeater (Biospec Products). Tubes were spun briefly to pellet the garnet mixture and reduce foam. The lysate was transferred to a new 1.5 ml tube using a syringe and 21 gauge needle, followed by passage through the needle 10 times to shear genomic DNA. Total RNA was then extracted using the Qiagen RNeasy Mini kit according to the manufacturer's instructions. Quality of the RNA was assessed by comparing the 28S:18S rRNA bands on a 1% agarose gel. RNA was used for these assays if the 28S band was more intense than the 18S band.

Reverse transcription

All samples including the SKBR3 cell line RNA's were individually quantified by the Beckman DU 640 spectrophotometer. One microgram of RNA from each sample was reverse-transcribed utilizing the Superscript™ First Strand Synthesis System for RT-PCR kit (Life Technologies). Each reaction mixture was in a volume of 20 μl and contained one microgram of RNA, 1 μl of random hexamers (50 ng/ μl), 2 μl of 10 \times RT buffer, 2 μl of 25 mM MgCl_2 , 1 μl of 10 mM dNTP mix, 2 μl 0.1 M DTT, 1 μl RNase inhibitor, 1 μl Superscript II RT (50 units/ μl), and H_2O needed to bring volume to 20 μl . Reactions omitting enzyme were used as negative controls.

Quantitative PCR

All primers were designed by Perkin Elmer Primer Express software, except for MGB1 which was designed by utilizing Primer3 program (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Amplification products were designed to cross introns to avoid amplification of genomic DNA. Beta-2 microglobulin (B2M) is ubiquitously expressed and was used to normalize expression. There has been no gene or set of genes found that are expressed at equivalent levels in all cells/tissues/cancers. We chose B2M for its robust expression in all samples tested. All primers were diluted to a stock concentration of 10 μ M and subsequently diluted to a working concentration of 900 nM, except for BU101 primers which were used at a concentration of 300 nM. (Table 1).

All quantitative PCR was performed on an ABI Prism 7700 Sequence Detection System (PE Biosystems, USA) using the ABI SYBR Green PCR master mix. Briefly, each 20 μ l cDNA reaction was diluted 1:20 and 5 μ l of this dilution was used in each PCR reaction (equivalent to 50 ng of the initial RNA). Serial dilutions of the SKBR3 cell line cDNA were used to construct standard curves for each PCR product. PCR conditions were as follows: 50 °C for 2 min followed by 95 °C for 15 min then 40 cycles of 95 °C for 15 s and 62 °C for 30 s. For each reaction, standard curves for both the gene markers and reference gene (B2M) were made. Relative amounts of each marker and reference gene were calculated by comparisons with standard curves. All PCR products were run on 3% agarose gels to confirm the specificity of the amplification. In all samples with a non-zero value measured by quantitative PCR, an amplification product of the appropriate size was visible by ethidium bromide staining after agarose gel electrophoresis.

Statistical methods

Pearson product moment correlations were derived using Statgraphics Plus 5.0. Normalized quantitative PCR values were compared to clinico-pathologic (tumor

size, stage, nodal status) and molecular parameters (steroid hormone receptor, p53, HER2-neu).

Results

Expression of epithelial and breast specific genes

We extracted total RNA from 95 separate primary breast cancers and 9 lymph node metastases (one derived from the same patient as one of the primary cancers) for a total of 104 separate breast cancer containing specimens from 103 patients. In addition, RNA was extracted from 10 benign breast specimens (from reduction mammoplasties) and 13 lymph nodes from patients who underwent lymphadenectomy for benign conditions. One μ g of RNA from each specimen was converted to cDNA by random priming using MuLV reverse transcriptase under standard conditions. Quantitative PCR was performed on each of the four breast markers (BU101, BS106, MGB1, and CK19) in the same assay. Also included on the 96-well plate were reactions to amplify β_2 Microglobulin to assure that PCR quality cDNA was present. In parallel, cDNA from the SKBR3 breast cancer cell line was also prepared. The SKBR3 line was found to have abundant expression of all mRNA's under consideration. Serial two-fold dilutions of SKBR3 cDNA were used to construct a standard curve for each PCR assay on the ABI 7700. Using the supplied software, values were derived in comparison to the standard curve.

An aliquot of each PCR reaction was electrophoresed to determine if a product of the correct size was present after amplification. In each case where the quantitative PCR values were higher than the baseline (no RT control), a specific product was detectable by ethidium bromide staining. The results of these assays are summarized in Table 2. The values are expressed in nanogram equivalents to the SKBR3 cell line used as a standard in all assays. For this calculation, a value of 50 is equal to the level in SKBR3 cells. For each marker, detectable expression varied over a range of five orders of magnitude (100,000 fold) likely indicative of the dynamic

Table 1. Primers sequences used in this study

Gene	Sequence of selected primer pairs	Length of amplicon	Measured Tm of amplicon
B2MF	5'-GCTGTGCTCGCGTACTCTCTTTCTGG-3'	295 bp	62 °C
B2MR	5'-GTCACATGGTTACACGGCAGGCATACAT-3'		
BU101F	5'-TTGCAGCCAAGTTAGGAGTG-3'	160 bp	62 °C
BU101R	5'-AAGACAGTGGAACCCAGGATG-3'		
BS106F	5'-AAGCCCCTGATGCTGAAACC-3'	181 bp	62 °C
BS106R	5'-TGCAGAAGACTCAAGCTGATTCC-3'		
CK 19F	5'-CCGCGACTACAGCCACTACTACAC-3'	169 bp	62 °C
CK 19R	5'-GAGCCTGTTCCGTCTCAAA-3'		
MammaF	5'-TCTCCCAGCACTGCTACGC-3'	82 bp	62 °C
MammaR	5'-TTAGACACTTGTGGATTGATTGTCTTG-3'		

Table 2. Results of quantitative RT-PCR in breast, lymph nodes, PBMC, and normal tissues

Type of Tissue	BU101 ^a	BS106	Mammaglobin	CK19
Normal Breast	10/10 (100%)	10/10 (100)	10/10 (100)	10/10 (100)
Range ^b	7-70	0.04-2500	0.2-16	0.02-2
Primary BrCa	82/95 (86%)	94/95 (99)	90/95 (95)	95/95 (100)
Range	0-1400	0-9400	0-130	0.05-80
Involved LN	9/9 (100%)	9/9 (100)	6/9 (67)	9/9 (100)
Range	1-260	2-5900	0-110	7-53
Normal LN	0/13 (0%)	1/13 (8)	0/13 (0)	3/13 (23)
Range	0	0-0.07	0	0-0.001
PBMC ^c	0/10 (0%)	10/10 (100)	4/10 (40)	6/10 (60)
Range	0	0.01-0.1	10 ⁻⁴ -10 ⁻³	10 ⁻⁸ -10 ⁻⁵
Normal Tissues ^d	6/10 (60%)	3/10 (30)	4/10 (40)	10/10 (100)
Range	3-3000	6-365	0.005-32	0.03-20

^a Number of tissues from each group that had detectable expression of the indicated gene by quantitative RT-PCR. Each reaction was electrophoresed to verify the presence of the appropriate sized product.

^b The range of expression in SKBR3 nanogram equivalents. A value of 50 is equal to the level of expression of that gene in SKBR3 cells.

^c Peripheral blood mononuclear cells from women with no evidence of cancer were collected from citrate treated blood collection tubes and total RNA extracted and analyzed.

^d Normal tissues included prostate, testis, pancreas, spleen, kidney (*n* = 2), skeletal muscle, colon, liver and stomach.

range of the assay. RNA's from all 10 of the normal breast samples were positive for each marker assayed.

Of the 95 primary breast cancers assayed, all were positive for CK19 with values ranging over 1600 fold. However, the three breast specific markers were not detected in every primary breast cancer. BS106 had detectable expression in all but one cancer (99% positive), Mammaglobin was undetectable in five cancers from this set (95%), and BU101 was undetectable in 13 of the samples (86%). The single primary cancer that had undetectable BS106 expression was also negative for BU101 and Mammaglobin. Histology of this cancer was of the ductal type with no obvious pathologic or molecular features distinguishing it from the remainder of the samples in this study. Of the nine lymph nodes with pathologically diagnosed breast cancer, Mammaglobin was undetectable in one third of them while the other markers were expressed in all of these samples. The range of expression in the primary cancers compared to the metastatic lymph nodes was comparable for each of the markers.

We also assayed levels of these mRNA's in a series of specimens from women without evidence of breast cancer including 13 normal lymph nodes and 10 buffy coat samples from peripheral blood. Relevant to detection of occult disease in axillary lymph nodes, low levels of CK19 and BS106 were detected in 3 and 1 of these RNA's respectively. The same lymph node with expression of BS106 was also positive for CK19. BU101 and Mammaglobin expression were undetectable in all 13 specimens. Relevant to detection of breast cells in the circulation analysis of peripheral blood mononuclear cells (unfractionated buffy coat derived from citrate collection tubes) we did detect CK19 (60%), BS106 (100%), and Mammaglobin (40%) in many of the peripheral blood lymphocyte samples. Given that these samples were derived from venipuncture, it is possible that skin

epithelia may have contributed to these signals, particularly for CK19. However, the consistent and relatively high expression of BS106 in the white blood cell fraction is likely indicative of a low expression of this gene in a specific subset of mononuclear cells. In addition, we assayed expression of these markers in a series of normal tissues including prostate, testis, pancreas, liver, stomach, colon, kidney, muscle, and spleen. As anticipated from the analysis of the Incyte database, we detected expression of the BS106, BU101, and Mammaglobin in the testis and prostate. Further, a high level of expression of Mammaglobin and BU101 was observed in skeletal muscle. BU101 was also expressed at relatively high levels in the colon and kidney contrary to expectations. Finally, all of these tissues had detectable levels of CK19 as expected.

A comparison of expression patterns in 40 individual primary tumor specimens is shown in Figure 1 arranged by increasing levels of BS106. Two of these samples demonstrate coincident lack of expression for both Mammaglobin and BU101. Previous work has shown that these markers are co-expressed in breast cancer and our current findings support this contention [6].

Clinical and molecular correlates of expression

We examined the expression levels of these genes and compared them to the known pathologic, clinical, and molecular parameters of the primary and metastatic breast cancers in this study. Pearson's product moment correlations were calculated pair wise for all variables. No correlation was observed between tumor size, histology, or presence of metastatic disease and the level of any of these markers. However, we did observe statistically significant correlations between the absence of steroid hormone receptors and increasing Mammaglo-

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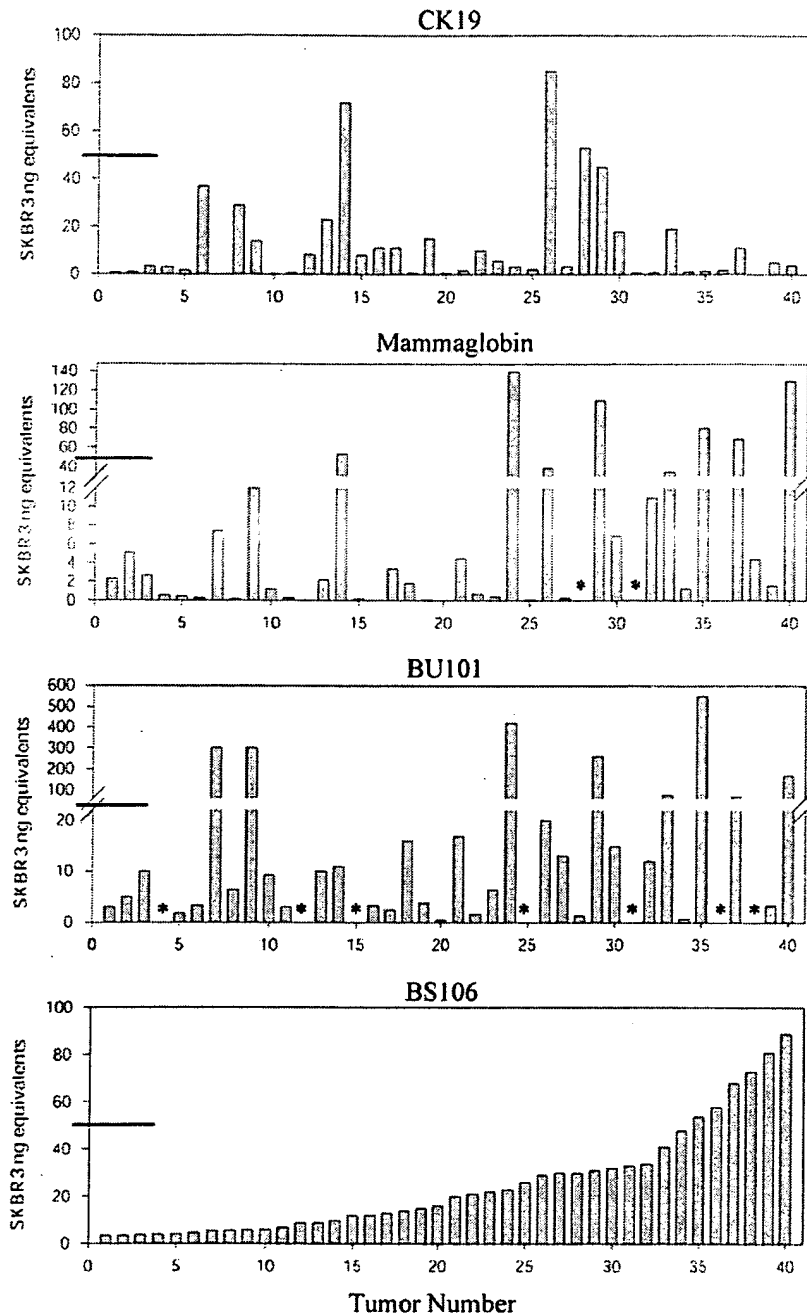


Figure 1. Expression levels of 40 representative primary breast cancers of the four genes examined in this study. The Y-axis values are in SKBR3 nanogram equivalents. A value of 50 (marked on the y-axis) indicates that a sample had the same relative level of expression as the SKBR3 line per amount of total RNA. Samples with (*) had no expression of the indicated gene. All other samples had specific and measurable expression values by quantitative RT-PCR as described.

bin expression ($p=0.02$). A stronger association was observed between high levels of HER2/neu protein expression (assessed by immunohistochemistry) and increasing amounts of the BS106 mRNA ($p=0.003$). Finally, the levels of BU101 and Mammaglobin showed a strong positive correlation as previously described ($p=0.0001$) [6].

Discussion

In the current study, we describe the expression pattern of two new genes that may be useful for early detection or monitoring of regional or systemic disease burden in breast cancer patients. These genes were originally found by *in silico* analysis of an expression database.

The criteria for this search included high level expression in most breast expression libraries (benign or malignant) and low or absent expression in libraries from other tissues. One of these genes (BU101) also described as Lipophilin B, is a member of the uteroglobin family [7] with unknown function. The other new gene (BS106) in this study contains sequence and post-transcriptional properties consistent with it being a mucin-like protein. Recently it was independently isolated also using an *in silico* approach and named "small breast epithelial mucin" [16].

To determine the utility of these potential breast-specific genes we compared expression to two established markers (CK19 and Mammaglobin) at the mRNA level in a series of frozen tissue specimens. Quantitative RT-PCR was performed on a total of 105 breast specimens (95 cancers and 10 benign) and 22 lymph nodes (9 with breast cancer and 13 from non-cancer patients). Wide variations in expression levels were observed for all types of samples. For each gene studied, we were able to detect expression over a range of five orders of magnitude corresponding to authentic PCR amplification products (as determined by gel electrophoresis). Other than CK19, no single marker was expressed in all breast cancer specimens. BS106 was detected in all but one of the malignant samples, however the one sample that was negative for this gene also had undetectable levels of Mammaglobin and BU101, indicating that a small subset of breast cancers may escape detection by this panel of breast-specific markers.

We observed a highly significant correlation between expression of Mammaglobin and BU101. This supports and extends previous work showing that these two genes are commonly co-expressed in the breast [6]. The protein products from these two members of the uteroglobin family form a heterotetramer in breast cells and the detection of this secreted product may also be a useful surrogate for measuring tumor burden. Antibodies to these proteins have been developed and are currently being characterized.

Our intent in characterizing new breast specific expression markers was to find targets that will be useful for early detection and monitoring of disease status. As such, the ability to detect a very high percentage of breast cancers is critical. Equally important is the capability of the markers to discriminate low levels of breast cancer cells among a vast excess of lymphoid cells (from lymph nodes, peripheral blood, or bone marrow). In a survey of non-cancer bearing lymph nodes, we found low expression levels of CK19 in over 20% of these samples while expression of the breast specific markers was at a much lower frequency.

Cytokeratin 19 has been the most widely used marker for detecting breast epithelial cells outside of the breast. Immunohistochemistry, PCR, nested PCR, and quantitative PCR have all been used to detect expression of this gene. CK19 is neither breast nor cancer specific, but it is highly expressed in most if not all epithelium with little or no expression in lymphoid cells. Therefore,

any specificity that this marker has resides in finding ectopic expression in sites such as lymph nodes, blood, and bone marrow. While this marker may have significant utility in detecting occult tumor in lymph nodes and bone marrow of breast cancer patients [20–22], studies performed in peripheral blood show a relatively high rate of false positives, both by immunohistochemistry and RT-PCR [23,24]. However, with more quantitative analysis, a higher degree of specificity has been achieved [25,26].

Unlike CK19, Mammaglobin is expressed predominantly in breast epithelial cells [14]. It is frequently elevated in breast cancer compared to normal breast epithelium making it an attractive marker for detection. Because it has a tissue restricted expression pattern, detection of Mammaglobin positive cells in circulation has a significantly higher positive predictive value than CK19 [24]. However, since not all primary cancers express this gene, it may have a lower sensitivity than the pan-epithelial cytokeratin marker. Results from the current study support this conclusion.

The addition of two newly discovered genes that are expressed in most breast cancers expands the range of possible markers for the design of diagnostic assays to detect and measure occult or minimal residual disease. For direct detection of breast cancer cells based upon the RNA expression of these genes, sensitive and specific RT-PCR approaches may be useful. In addition, both BU101 and BS106 appear to code for secreted proteins adding to the potential utility of these markers.

Declaration of competing interests

This work was done as a collaborative effort between scientists at Duke University Medical Center and Abbott Laboratories in the context of an NIH funded grant to discover novel biomarkers for breast cancer detection. Intellectual property related to the two new breast biomarkers described in this study is owned by Abbott Laboratories.

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Review

Mammaglobin: a candidate diagnostic marker for breast cancer

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Abstract

Mammaglobin, known for its mammary tissue specificity, has been discussed as a promising diagnostic marker in breast cancer for almost 10 years. In particular, the application of mammaglobin RT-PCR to detect disseminated breast cancer cells has been reported. More than 25 publications evaluate the detection of mammaglobin mRNA in lymph node, blood, and bone marrow specimens of breast cancer patients. Recently, structural details about the mammaglobin complex have been discovered, and these findings can be implemented to optimize detection of the secreted protein. This review summarizes the findings of almost 50 published studies and the current knowledge about the diagnostic utility of mammaglobin.

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Keywords: Mammaglobin; Secretoglobin; Breast cancer; Diagnostic marker; RT-PCR; Serum

Introduction

Human mammaglobin (h-MAM) was identified by Watson and Fleming [1,2] in 1996 using differential display PCR technique. Mammaglobin belongs to the uteroglobin/Clara cell protein family of small epithelial, secretory proteins, which has recently been named secretoglobins with currently 23 known family members [3,4]. The founding family member, uteroglobin, was discovered as the major protein component of rabbit uterine secretions [5] and was the first progesterone-regulated mammalian protein discovered. All six human member genes are localized on chromosome 11q12.2 and form a dense cluster [4]. Mammaglobin has been found in breast epithelial cells and is overexpressed in breast cancer [2]. Due to its breast-specific expression, mammaglobin mRNA and protein are of high interest as candidate diagnostic markers for breast cancer. With over 50 publications on its use since the year 2000, it is considered the most promising molecular marker for breast cancer to date.

Expression in primary tumors

Mammaglobin expression was first studied by Watson and Fleming [2] in 35 breast carcinomas using RT-PCR and Northern blot analysis. They reported overexpression of mammaglobin (at least 10-fold relative to normal breast tissue by Northern blot hybridization) in 23% of the tumors tested with no correlation to a specific histology. In a subsequent study, no gene amplification or gene rearrangement was found in tumors overexpressing mammaglobin [6], indicating changes in transcriptional regulation. The same group later studied mammaglobin protein expression by immunohistochemistry and detected strong reactivity in 81 out of 100 breast tumors [7] independent of stage and histological type of the cancers. In this study, mammaglobin mRNA was found for the first time in lymph nodes demonstrating the utility of mammaglobin-directed RT-PCR for detection of disseminated tumor cells. Leygue et al. [14] found mammaglobin expression in tumor epithelial cells, but not in stromal or inflammatory cells, analyzing 13 breast tumor tissues using *in situ* hybridization.

Recently, Nunez-Villar et al. [8] evaluated an extensive panel of 128 breast cancer specimens by RT-PCR. A correlation between high levels of mammaglobin and expression of estrogen and progesterone receptor, diploid DNA content, low Ki67 labeling index, low nuclear

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grade, and the absence of axillary nodal invasion was found. Interestingly, these findings indicate that elevated mammaglobin expression in breast cancer is associated with a less aggressive tumor phenotype.

Expression in other tissues

Initially, Fleming and Watson [9] examined mammaglobin expression using an extensive panel of normal poly-A-selected mRNAs from pooled populations of adult and fetal human tissues. Mammaglobin mRNA was only detected in mammary gland using a commercially prepared dot-blot. In concordance, Zach et al. [10] did not detect mammaglobin expression using RT-PCR in human uterus, ovary, or leukocytes. However, another group reported mammaglobin expression in benign and malignant tissue from the ovary, uterus, and cervix using a nested RT-PCR approach [11]. The same study described preliminary results using quantitative real-time PCR, indicating significantly higher mammaglobin expression in mammary tissues than in ovarian and endometrial tissues. In addition, all peripheral blood and bone marrow samples from healthy volunteers in this study ($n = 124$) tested mammaglobin negative. Recently, the detection of mammaglobin expression in the secretory coil of the eccrine sweat glands of human skin was reported [12].

Lymph node metastases

The detection of mammaglobin expression by RT-PCR also has been implemented to detect occult metastases in lymph nodes of breast cancer patients. The presence of lymph node metastases is considered the most important prognostic indicator for breast cancer. RT-PCR has been suggested to provide higher sensitivity to detect disseminated tumor cells in comparison to conventional lymph node staging approaches. The first report of the use of mammaglobin as a nodal metastases marker was made by Min et al. [13]. They compared seven marker genes for their potential application in lymph node analysis by testing a panel of breast cancer cell lines and normal lymph node specimens using RT-PCR. Mammaglobin and carcinoembryonic antigen (CEA) expression were not detected in normal lymph node tissues in contrast to cytokeratin 19 (CK19), mucin 1 (MUC-1), maspin, vascular endothelial growth factor (VEGF), and transforming growth factor- β (TGF- β). Mammaglobin was detected in all breast cancer cell lines tested, whereas CEA was found only in a subset. Subsequently, Leygue et al. [14] demonstrated detection in 13 out of 13 histology-positive breast cancer lymph samples by mammaglobin RT-PCR, whereas no mammaglobin expression was detected in lymph nodes from cases without histologically detectable tumor cells.

In addition to mammaglobin, mammaglobin B (MGB2) was also reported to detect 100% of the histology-positive lymph node samples tested ($n = 14$). Mammaglobin B was identified as a highly homologous gene to mammaglobin; however, the expression of both genes is non-concordant in nonmalignant and neoplastic tissue [15]. The same group also proposed the use of mammaglobin B as a marker for detection of micrometastasis in lymph nodes of breast cancer [16] and various abdominal cancers [17], indicating a lack of mammary gland specificity for mammaglobin B.

New high-throughput RT-PCR protocols could be particularly powerful for intraoperative sentinel lymph node analysis. In contrast to axillary lymph node dissection, sentinel lymph node biopsy (SLNB) implements mapping of the primary tumor draining lymph node. SLNB therefore can provide prognostic information regarding metastatic spread with minimal associated morbidity. Kataoka et al. [18] reported for the first time the analysis of 70 stage I and II breast cancer sentinel lymph nodes using mammaglobin RT-PCR. This study also demonstrated increased sensitivity of RT-PCR in comparison the conventional histology by H&E (hematoxylin and eosin staining).

The sensitivity of mammaglobin RT-PCR was confirmed by comparison with other potential mRNA markers (CK20, CEA, prostate-specific antigen (PSA), melanoma-associated antigen 1 (MAGE1), and MAGE3) by Ooka et al. [19]. Mammaglobin expression was also detected in some histology-negative lymph node specimens of breast cancer patients but not in normal lymph node specimens. It can be concluded that mammaglobin RT-PCR might detect breast cancer micrometastases missed by conventional histology. Marchetti et al. [20] compared the application of mammaglobin and CEA in lymph node samples from 248 patients. Mammaglobin was detected in 97% of lymph node samples with histologically documented metastasis and in 29% of lymph nodes without histological evidence of metastatic disease. In comparison, CEA was detected in 79% of the lymph nodes with and in 20% of the lymph nodes without confirmed metastatic disease.

Although mammaglobin is a good marker in itself, several studies suggested the combination of multiple markers for breast cancer lymph node analysis [21–23]. Using real-time RT-PCR, Mitas et al. [22] evaluated the expression level of 12 cancer-associated genes in 51 negative-control lymph nodes and in 17 histopathology-positive axillary lymph nodes. Receiver operating characteristic (ROC) analysis revealed mammaglobin as the most accurate marker for lymph node metastasis. Our group established a real-time multigene RT-PCR assay detecting expression of mammaglobin and three complementary transcribed genes, B305D, B726P, and GABA π [23]. ROC curve analysis demonstrated perfect sensitivity and specificity for the multigene assay on a panel of 50

metastatic breast cancer and 27 normal control lymph node specimens. An approach using mammaglobin alone or in a multiplex assay will likely be a powerful tool in the detection of occult lymph node metastases and will provide a good prognostic indicator for breast cancer patients.

Detection in blood, bone marrow, and leukapheresis samples

The detection of circulating mammary carcinoma cells in peripheral blood specimens of breast cancer patients using mammaglobin RT-PCR was first reported by Zach et al. [10]. In this study, 114 breast cancer patients were screened using a nested reverse transcriptase polymerase chain reaction (RT-PCR) assay with a calculated detection sensitivity of one tumor cell per 10^6 – 10^7 white blood cells. Peripheral blood samples of 25% of the breast cancer patients were positive for mammaglobin mRNA, with 28% positive at time of diagnosis, 6% positive with no evidence of disease, and 49% positive with metastatic disease. A correlation to clinical stages of disease, CEA plasma level, and estrogen receptor status was also found. Later, the same group reported the analysis of 286 breast cancer blood specimens with 43% mammaglobin positives in metastatic disease patients [24]. A stochastic model was developed to validate the results obtained with the reported nested RT-PCR assay [25]. The relationship between number of positive PCR setups per blood sample, patient status, and relapse rate of breast cancer patients was demonstrated. Four percent of patients with benign breast lesions were reported to be mammaglobin positive but with a lower amount of signal (as determined by number of positive PCR setups) in comparison to breast cancer patients.

Fleming and Watson [9] found mammaglobin mRNA by single-step RT-PCR amplification followed by hybridization to radiolabeled mammaglobin cDNA in 9 out of 15 (60%) peripheral blood stem cell collections from leukapheresis products of breast cancer patients. Suchy et al. [26] reported the detection of mammaglobin expression in only 11/98 blood samples of breast cancer and 3/12 of ovarian cancer patients using a real-time Taqman RT-PCR approach testing mononucleated cells from 5 ml of peripheral blood. Sensitivity might have been compromised in this study by the small amount of blood (150 μ l) tested in each reaction. Sequence specificity, particularly in comparison to mammaglobin B, could not be verified due to missing primer and probe information in this publication. PCR cross-reactivity with mammaglobin B RNA could be a main reason for loss of specificity. Gruenewald et al. [27] demonstrated the use of mammaglobin to detect circulating breast cancer cells by nested RT-PCR with a sensitivity of one tumor cell per million mononuclear blood cells. Mammaglobin was shown to be

more specific and sensitive than epidermal growth factor receptor (EGF-R) and cytokeratin-19 testing 145 breast cancer and 51 control blood samples.

Controversially, the induction of mammaglobin expression in cell lines and in bone marrow and peripheral stem cells from patients without epithelial cancer by several cytokines has been reported [28]. These findings raise the

Table 1a
Summary of published mammaglobin expression studies on breast tumors and lymph nodes

Reference	N	Approach	Data
Breast tumor			
[2]	35	Northern blot hybridization	23% with strong signal
[7]	100	IHC; RT-PCR	81% strongly positive
[14]	13	In situ hybridization; RT-PCR	80% positive
[9]	100	IHC, Northern analysis	80% positive
[23]	27	Real-time multigene RT-PCR assay (MG, B305D, B726P, and GABA π)	63% MG positive, 100% multigene positive
[34]	30	Several nested RT-PCR assays	97% MG positive
[8]	128	Semiquantitative RT-PCR	High MG in less aggressive tumor phenotype
[47]	174 and 21 benign	RT-PCR	60% tumors positive and 43% benign
Axillary lymph nodes (ALN)			
[7]	11 mets	IHC; RT-PCR	91% positive
[14]	9 mets	In situ hybridization; RT-PCR	100% positive
[9]	21 breast, 12 other	IHC; Northern analysis	43% breast mets positive
[19]	177 from 17 BC patients	RT-PCR (CK20, MAGE1 + 3, CEA, PSA, MG, and MG-B)	MG signal in 14/14 mets + 49/163 histology negatives
[13]	20 normal ALN	RT-PCR (CK19, MUC-1, maspin, VEGF, TGF- β , CEA, and MG)	All MG negative
[20]	248 BC patients	RT-PCR (MG and CEA)	MG signal in 86/89 mets + 46/159 histology negative
[22]	17 mets, 51 normal	RT-PCR (PIP, MG-B, CEA, CK19, VEGF, erbB2, muc1, c-myc, p97, vim, and Ki67)	In ROC curve analysis MG most accurate marker
[23]	50 mets, 27 normal	Real-time multigene RT-PCR assay (MG, B305D, B726P, and GABA π)	80% MG and 100% multigene positive
Sentinel lymph nodes (SLN)			
[18]	66	CEA and MG RT-PCR	ALN status prediction with 98.5% accuracy
[21]	146 from BC 123 patients	RT-PCR	78% sensitivity + 86% specificity SLN mets detection

IHC, immunohistochemistry; MG, mammaglobin; BC, breast cancer.

possibility of false-positive results in normal donor specimens, which have not been supported by other studies so far. Silva et al. [29] tested 47 peripheral blood samples, 15 bone marrow aspirates, and 28 blood progenitor cell samples from normal control donors and did not detect mammaglobin transcripts using nested RT-PCR. Mammaglobin expression was detected in 19/78 breast cancer specimens from patients before chemotherapy and 5/30 samples of breast cancer patients during treatment. Six out of seven bone marrow aspirates from patients with metastatic disease were also reported positive.

To achieve higher sensitivity to detect circulating tumor cells in peripheral blood, positive (epithelial cell capture) or

negative (CD45⁺ cell depletion) enrichment protocols can be implemented. Our group demonstrated the application of Taqman real-time mammaglobin RT-PCR to detect 62% epithelial cell enriched blood samples from breast cancer patients [30]. By adding the detection of additional complementary transcribed genes, 84% of the samples were detected. Interestingly, a comparison of mammaglobin mRNA expression in peripheral blood and serum tumor markers (carcinoembryonic antigen CEA and CA 15.3) in 33 metastatic breast cancer patients was reported [31]. Mammaglobin RT-PCR detected 54% of the samples tested. The combination of mammaglobin mRNA detection with CEA or CA 15.3 increased the sensitivity to 81% and 90%,

Table 1b

Summary of published mammaglobin expression studies in bone marrow, blood, and other tissue types

Reference	N	Approach	Data
<i>Bone marrow</i>			
[33]	111	Real-time RT-PCR (LighCycler™)	29.7% positive: 24% of this group and 7.7% of negative patients developed distant mets
[34]	7	Several nested RT-PCR assays (CEA, CK19, CK20, Muc-1, EGFR, maspin, and MG)	43% MG positive
[35]	60 patients (and/or blood)	Nested RT-PCR	23.7% with and 9% with no MD positive; control samples (n = 48) negative
[29]	7	Nested RT-PCR	6/7 from MD patients positive
<i>Blood</i>			
[10,24,25]	114, 286, 610	Nested RT-PCR	114 patients: 6% NED and 49% MD; 286 patients: 3% NED and 43% MD; 610 patients: 15% NED and 54% MD positive
[26]	98	Taqman real-time PCR	11/98 of BC, 3/12 ovarian cancer patients and 0/21 healthy female volunteers positive
[29]	108	Nested RT-PCR	19/78 before chemotherapy, 5/30 on treatment positive
[31]	33	RT-PCR and serum tumor markers	54% samples positive. Combined with CEA or CA 15.3: sensitivity at 81% and 90%
[32]	106	Nested RT-PCR	12% stage 0, 25% I, 32% II, and 33% stage III; 54% MD and 0/27 controls positive
[27]	133	Nested RT-PCR (MG, CK-19, and EGF-R)	0/31 controls, 0/20 hematological disorders, 0/12 DCIS and 11/133 invasive BC positive
[30]	32	Enriched epithelial cells (Dynal immunomagnetic). Taqman real-time RT-PCR	20/32 MG positive. 27/32 specimens positive for MG or complementary transcribed genes
[34]	37 leukapheresis, 5 blood	Several nested RT-PCR assays (CEA, CK19, CK20, Muc-1, EGFR, maspin, and MG)	0% peripheral blood and 8% leukapheresis samples MG positive
[7]	15 leukapheresis	Nested RT-PCR followed by Southern hybridization	9/15 patients and 0/4 normal donors positive
<i>Other tissue/sample types</i>			
[9]	7 human BC cell lines	RT-PCR (CK19, MUC-1, maspin, VEGF, TGF- β , CEA, and MG)	All BC cell lines positive for MG
	Other tissues	Extensive panel of normal human tissues mRNAs	MG detected only in mammary gland
[2]	15 Other tissues	Northern blot and RT-PCR analysis	MG only in normal breast and BC
[11]	73 normal + 79 malignant epithelial and 33 normal mesenchymal tissues	Nested RT-PCR	ovary, uterus, cervix, and prostate samples positive. 0/124 control blood and BM positive
[12]	25 other tissues	Real-time RT-PCR, in situ, IHC	Expression in skin secretory coil of sweat glands

MD, metastatic disease; NED, no evidence of disease; DCIS, ductal carcinoma in situ.

respectively, suggesting mammaglobin mRNA as a potential adjunct to routinely used serum markers. Recently, the same group evaluated the correlation between mammaglobin expression in peripheral blood and known prognostic factors for breast cancer patients [32]. Mammaglobin mRNA expression frequency was shown to be increased in patients with unfavorable prognostic factors (tumor size and stage); however, no significant differences could be confirmed.

Mammaglobin RT-PCR has also been implemented for the detection of bone marrow micrometastasis in breast

cancer patients. One study analyzing 111 bone marrow samples demonstrated that patients with both bone marrow, and lymph node metastases showed significantly higher risk of recurrence than those with either one of them or none [33]. A later study reported the detection of 43% of breast cancer bone marrow specimens using nested mammaglobin RT-PCR but no signal in samples from control donors [34]. Other molecular markers tested (CK19, MUC-1, CEA, and EGFR) were also detected in control bone marrow, peripheral blood, and leukapheresis samples, indicating insufficient specificity of these markers. A study testing bone marrow and peripheral blood samples of 60 breast cancer patients by nested mammaglobin RT-PCR [35] demonstrated higher percentage of positive cases among patients with metastasis and higher in patients with progressive disease than in those responding to therapy. Summaries of the findings from the studies mentioned above are in Tables 1a and 1b. The primers used for PCR detection are listed in Table 2.

Mammaglobin protein

While the use of nucleic acid markers such as mammaglobin mRNA is being developed as an increasingly more important method of diagnosis, detecting the protein complex by a sensitive assay could have many advantages: The traditional ELISA format has been implemented in many laboratories already and could be adapted specifically for this complex; the secreted complex is more likely to be circulating in the blood secreted from small tumors than are circulating tumor cells in earlier stages of disease; and the make-up of the protein complex in the blood may yield more information than the message alone.

To develop specific and sensitive diagnostic agents targeting mammaglobin, one must understand the protein's structure. Mammaglobin is predicted to be a member of the uteroglobin family with homologues such as the rat prostatein C protein, the pig pheromaxin C, hamster heteroglobin B2, and the feline major allergen, FelD (see also Fig. 1). Conceptual translation of the gene product predicts a 93 amino acid polypeptide (M.W. = 10,498) with a putative leader sequence of 19 amino acids that is in fact cleaved during secretion of the native protein.

Uteroglobin family members tend to be homo- and heterodimers of either two uteroglobin-like molecules or a mammaglobin-like molecule bound to a lipophilin-B-like molecule [36–38]. Mammaglobin forms a covalent complex with lipophilin B via disulfide bonds and may exist as a dimer of covalent heterodimers in solution [39]. There are two predicted N-linked glycosylation sites, both of which may be linked to carbohydrate chains of about 3 kDa making carbohydrates account for about one third of the molecular mass of the full complex. Using mass spectrometry, masses of 23.4 and 16.2 kDa were determined for the fully glycosylated and the deglycosylated complexes, re-

Table 2
Primer sequences used in published mammaglobin RT-PCR studies

Study	Primer sequence
[2]	F-5'cagcggttccttgccttgc; R-5'ataagaagagaagggtgg
[14]	MG-sense 5'-ccgacagcagcagcctcac; MG-antisense 5'-tccgtagttgggtttctac
[8]	Outer primer pair: Zach et al. [10]
[47]	Min et al. [13]
[19]	F-5'ctctggctgcccctattgga; R = Watson and Fleming [2] MGB-F-5'actcctggaggacatggtga; MGB-R-5'tctgagccaacgccttgggt
[13]	Watson and Fleming [2]
[20]	Min et al. [13]
[22]	F-5'cgatgaactctgagcaatgt; R-5'ctgcagtctgtgagccaaag
[23]	Houghton et al. [30]
[18]	Min et al. [13]
[21]	Watson et al. [7]
[33]	F = Ooka et al. [19]; R = Watson and Fleming [2]
[34]	Min et al. [13] Inner-F-5'gagtcacagcagcaatgcc; inner-R-5'ccgtagttggtttcaccat
[35]	Outer-sense + outer-antisense = Watson and Fleming [2] Inner-sense-5'caccgacagcagcagcctca; inner-antisense-5'aatccgtagttggtttctca
[10,24,25]	MG-1-5'gaagttgctgtaggtctcctcatgctggc; MG-2-5'ctcaccataccctgcagttctgtgagc MG-3-5'ctccagcactgctacgcaggctc; MG-4-5'caectcaacattgctcagagtttcatccg
[26]	Primer and probe sequences of the assay used were not reported
[29]	Zach et al. [10]
[31]	Gruenewald et al. [27]
[32]	Zach et al. [10]
[27]	Outer primer pair = Watson and Fleming [2] Inner-F-5'tgaacaccgacagcagcag; inner-R = MG-antisense (Leygue et al. [14])
[30]	F-5'tgcatatgtaattgcaataaacctca R-5'tgcatatgtaattgcaataaacctca Probe-5'tcttaaccaacggatgaaactctgagcaatg
[9,7]	Outer-F = Watson and Fleming [2]; outer-R-5'tagcaggtttcaacaattgtc Inner-F-5'agcactgctacgcaggtctc; inner-R = Watson and Fleming [2]
[11]	Inner-F + inner-R = Gruenewald et al. [27] For paraffin-embedded tissues different primers were used because of partial RNA degradation: Outer-F2-5'tgcatatgtaattgcaataaacctca; outer-R2 = inner-R; inner-F2 = Mitas et al. [22]; inner-R2 = inner-R
[12]	F-5'caagacaatcaatccacaagtgtctaagac; R-5'cagagtttcatccgttgggttaagaaaacattc Probe-5'tcatctatggcattttagtggcattgtcgtctatgaactcttgaagaag

spectively [40]. The predicted mass for mammaglobin protein is 8480.3 Da; for lipophilin B it is 7688.1 Da and for the complex without glycosylation the predicted mass is 16,164.4 Da.

Molecular modeling has been used to investigate the putative structure of the mammaglobin complex (see also Fig. 2). In this model, each protein forms four alpha helices that sandwich in a head-to-tail orientation creating a hydrophobic core. This orientation allows for the formation of three disulfide bonds, which are in close proximity in the three dimensional model. Consistent with this model, the predicted helical secondary structure overlaps with the location of the helices in the model. CD data showing high alpha helical content have been measured, and limited peptide mapping has confirmed that the N-terminal cysteine of mammaglobin is covalently linked to the C-terminal cysteine of lipophilin B. The hydrophobic core may be capable of binding steroid-like molecules in the reduced state similar to the binding observed for other uteroglobin members [37,41,42]. The N-linked glycosylation sites are on opposite ends of the complex and in flexible loop regions between alpha helices. This probably allows for the large carbohydrate chains to extend in either direction into the solute.

When designing a molecular test for mammaglobin protein, the structural features mentioned above will have to be taken into account. Antibodies made to recombinant mammaglobin from *Escherichia coli* bind very weakly to the native protein (Houghton R., personal communication). This is likely due to steric hindrance by the carbohydrates extending over the small mammaglobin protein core. Antibodies would have to be made specifically to exposed protein portions or to the carbohydrate itself. This may be an advantage as looking at the various states of glycosylation or levels of free mammaglobin may greatly enhance the amount of information one could gain about the presence of a tumor, or the tumor's state-glycosylation patterns are known to change during transformation of a cell (e.g., Ref. [43]) and lysis of tumor cells could be releasing mammaglobin without glycosylation. Therefore, a diagnostic antibody that recognizes variations on mammaglobins

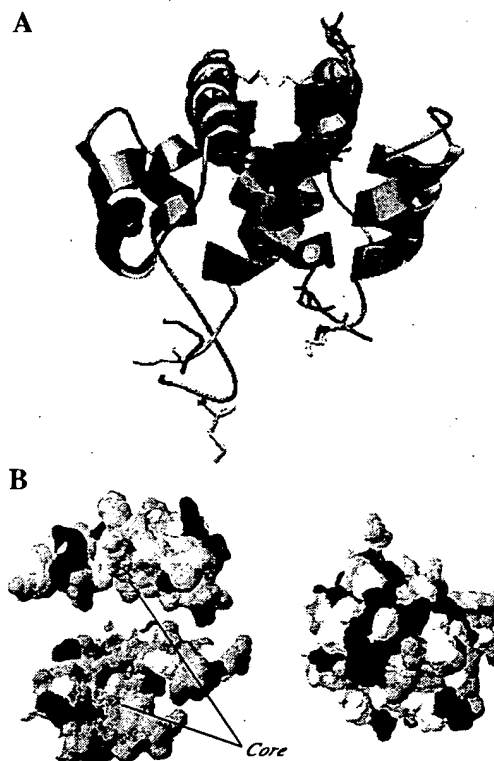


Fig. 2. 3D model of the mammaglobin protein complex. (Panel A) Ribbon diagram of the protein complex. Mammaglobin is in red and lipophilin B in blue. One can see the arrangement of four alpha helices per protein that neatly stack in an offset manner to allow for the six cysteines to come in close proximity (yellow stick models). Also shown are the two N-linked glycosylation sites (green sticks) at the beginning and end of helix three of mammaglobin that are predicted to allow for the attached sugars to point into the solvent at opposite poles. (Panel B) Surface plot colored by amino acid polarity. The surface diagram shows acidic residues in red, basic residues in blue and polar ones in yellow. Hydrophobic residues are in gray. The image on the right demonstrates the polar nature of the exterior of the folded protein. If the individual chains are moved apart and rotated on can see a hydrophobic pocket inside the protein (left image). This may be the binding pocket for the steroid-like ligand postulated to bind to these protein family members. Models were built using the Swiss-PdbViewer [49] and rendered using POV-Ray (<http://www.povray.org>).

Mammaglobin family members

Mammaglobin	---GSGCPLLENVISKINPOVSKTEYKELLQEFIDDNATTNAIDELKECFNLQTDETLSNVEVFMQLIYDSSLCDLF	75
Mammaglobin-B	---DSGCKLEDMVEKTIINSIDISIPEYKELLQEFIDSDAAAEAMGKFKQCFLNQSHRTLKNFGLMMHTVYDSIWCNMKSN	77
Prostatein-C3	---SGSGCSILDEVIRGTINSTVTLDHYMKLVKPYVDHFTKAVKQFKQCFLDQTDKTLNENGVMMEAIFNSESQQPS	77
Pheromaxein-C	---GSGCSYLERVISDTSDDSVTTDVYLASLQEIYSSDDTTQAIKELRECFNLQSEETLENFSVFMQVTVNSKLCAPF	75
	*** * * * * * * * * * * * * * * * * * *	

Lipophilin-B family members

Lipophilin-B	-EFCPALVSELDFFFISEPLFKLSLAKFDAPPEAFAAKLVKRCQTDQMSLQKRSLIAEVLVKI-LKKCSV	69
Lipophilin-A	--VCQALGSEITGFLLAGKPVFKFQAKFKAPLEAFAAKMEVKKCVDTMAYEKRVLITKTGKI-AEKCDR	68
Prostatein-C1	SQICELVAHETISFLMKSEELKKELEMYNAPFAAEAKLEVKRCVDQMSNGDRLVVAETLVYI-FLKCDVQWVETYYPEIDFYDMN	88
Pheromaxein-A	-IVCRALVKEPSAFLWKPDIEYKPELEFGAPPEAVDAKMKVKQCANGISFKKKILLTKTLVEILVKKCGFEDVKTLPDPSLGLSASVFK	90
	* * * * * * * * * * * * * * * * * *	

Fig. 1. Alignment of mammaglobin-like protein sequences and lipophilin B-like protein sequences. Sequence alignment of various sequences of these uteroglobin family members show two families that likely bond into heterodimers via the three cysteines that are highly conserved in each (gray shading). Interestingly, while each mammaglobin like member has at least one predicted N-linked glycosylation site (bold), none of the lipophilin B members do. Sequences were aligned using the CLUSTAL W algorithm [48].

carbohydrate chain or that is specific to free mammaglobin protein could add to the value of mammaglobin as a breast tumor marker by showing different stages of transformation or being more sensitive to early tumors that are secreting the protein without circulating in the blood themselves.

Protein-directed diagnostic approaches for mammaglobin

The presence of mammaglobin protein complex can be exploited in different ways for diagnostic assays: One could detect the protein directly, detect immune cells reacting to the protein, or look for circulating antibodies that recognize the protein. Using monoclonal antibodies directed against recombinant mammaglobin, Fleming and Watson [9] reported for the first time that secreted mammaglobin protein was detectable in sera of breast patients. In this study, sera samples from 25 normal and 135 patients with known breast cancer disease were analyzed. Only 1 normal sample yielded a positive result, whereas 33% of the primary breast cancer sera and 44% of the metastatic breast cancer sera tested positive. Later, our group reported the detection of elevated mammaglobin protein in sera of 14/30 patients with primary breast cancer, 16/36 with metastatic breast cancer, and 1/15 of normal female sera samples using antimammaglobin antibodies generated with recombinant purified protein in a capture ELISA format [44].

The mammaglobin protein complex from breast tumors also appears to be immunogenic and elicit mammaglobin-reactive CD8⁺ and CD4⁺ T cells. Jaramillo et al. [45] reported that the frequency of these cells in breast cancer patients is significantly higher than that observed in healthy female controls. If the presence of the CD4⁺ and CD8⁺ cells is an indicator of the body mounting an antitumor immune response, the amount of these cells could possibly correlate with disease outcome and recurrence. Antibodies to the complex are also found in the sera of breast cancer patients. Our group has tested for antibodies to the complex and its components and found a high degree of correlation between the disease stage and the levels of antibodies to a component of the mammaglobin complex, lipophilin B [46]. Mammaglobin-specific antibody levels were much lower, likely due to its being highly glycosylated. While the use of mammaglobin protein-directed assays is still in its infancy, approaches utilizing more specific anti-native complex antibodies and assays may provide even more information on diagnosis and disease prognosis in the future.

Conclusions

Mammaglobin could become an important tool for detecting primary or metastatic breast cancer, monitoring lymph nodes during and after surgery, and predicting disease outcome and recurrence. However, the clinical

relevance and application of mammaglobin specific assays still has to be evaluated in larger, prospective studies. The prominence of mammaglobin message in primary breast tumors and lymph nodes has been well established, and the specificity of multiplex assays that include mammaglobin is unsurpassed in breast cancer. It is still uncertain if circulating micrometastatic cells can be detected before the development of clinical disease, but mammaglobin RT-PCR in peripheral blood could be used to monitor adjuvant treatment efficacy. A recent study of our group (publication in process) indicates significant correlation between mammaglobin expression in blood and disease progression during chemotherapy. In addition, mammaglobin RT-PCR could be used to detect bone marrow micrometastases as a predictive marker for recurrence and as a potential selection marker for adjuvant chemotherapy. In view of the promising results of so many diverse studies, mammaglobin has many different potential applications in the future of breast cancer related diagnosis.

Acknowledgments

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Human Multiple Tissue Expression (MTE™) Array

76 human poly A⁺ RNAs arrayed on one nylon membrane



- Accurately profile gene expression in a broad range of tissues and cancer cell lines
- Whole-body imaging—access a large collection of human tissues and cell lines
- Featuring CLONTECH's Poly A⁺ Premium RNA™—for exceptional quality

CLONTECH introduces the **Human Multiple Tissue Expression (MTE™) Array**, an array of poly A⁺ RNAs for quickly and accurately profiling gene expression. The MTE Array combines the 50 tissue-specific poly A⁺ RNAs from the Human RNA Master Blot™ (1) with 26 new poly A⁺ RNAs from **cardiovascular and digestive tissues and cancer cell lines** (Figure 2). Since their introduction in 1996, our Master Blots have been referenced in numerous journal articles. When you use blots with a proven track record, you save time and get reliable results.

The MTE Array generates a snapshot of gene expression in all tissues—a virtual "whole-body image" in one easy hybridization experiment. The MTE Array also provides the quickest quantitative, tissue-specific gene expression profiles, thus allowing you to hypothesize about gene function. Each array arrives ready for immediate use with either radioactively or non-radioactively labeled probes. In addition, the MTE Array is economical, as it can be stripped and reprobed multiple times.

Access hard-to-obtain poly A⁺ RNAs

CLONTECH's Human MTE Array provides easy access to poly A⁺ RNAs from the widest range of tissues and cancer cell lines available. Normally, special facilities and permits would be required to obtain all 76 poly A⁺ RNAs included on the MTE Array. Furthermore, the time and cost required to prepare poly A⁺ RNA from these tissues would be prohibitive to most laboratories. The MTE Array is an extremely convenient and accessible tool for analyzing gene expression.

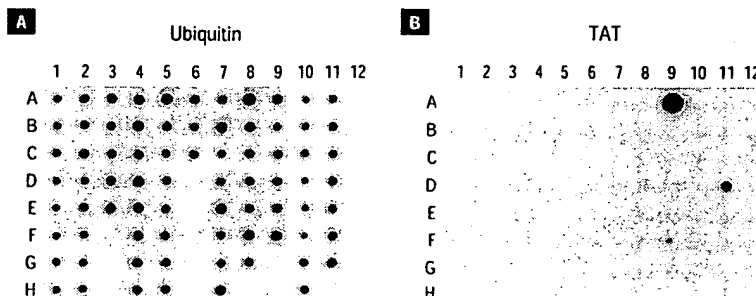
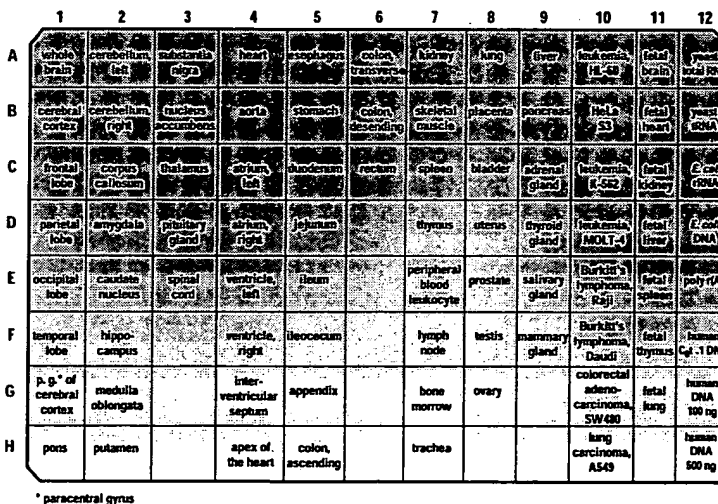


Figure 1. MTE™ Arrays reveal accurate gene expression profiles. Panel A. Hybridization with the Human Ubiquitin cDNA Control Probe. Panel B. Hybridization with a liver-specific cDNA probe for TAT. See Figure 2 for the identity of each dot. Arrays were hybridized overnight with radioactively labeled, randomly primed cDNA probes using ExpressHyb™ Hybridization Solution. Arrays were washed with 2X SSC, 1% SDS at 65°C, and 0.1X SSC, 0.5% SDS at 55°C, and were exposed to x-ray film at -70°C overnight.



* paracentral gyrus

Figure 2. CLONTECH's MTE™ Array. Columns 1-11: Poly A⁺ RNA samples. Column 12: Controls. Poly A⁺ RNA is applied to each MTE Array under alkaline conditions (10 mM NaOH) by a robotic pipetting station. The membrane is neutralized, and the poly A⁺ RNA is crosslinked to the membrane by exposure to ultraviolet light. Actual membrane size is 82 x 127 mm.

Human MTE™ Array...continued

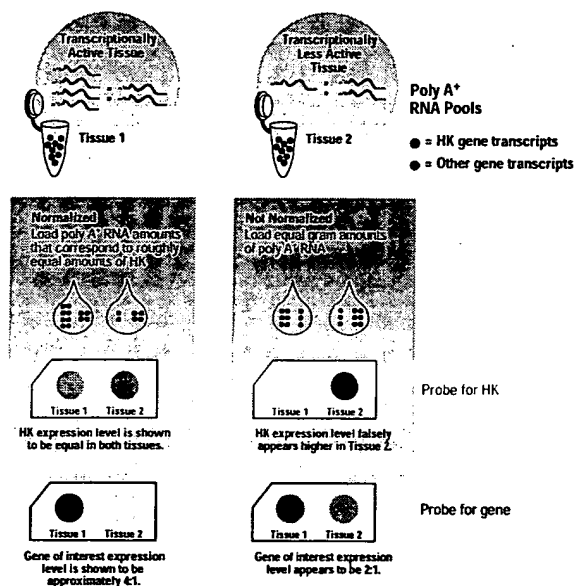


Figure 3. Normalization provides accurate results. As shown, normalized loading of poly A⁺ RNA results in more accurate quantification of gene expression. HK = housekeeping gene.

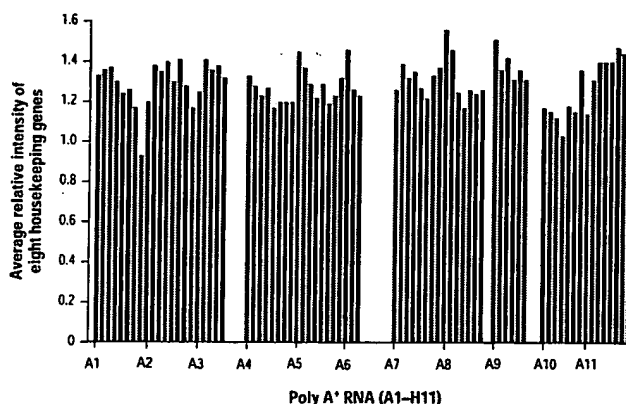


Figure 4. Average relative intensity of eight housekeeping genes after normalization of the MTE™ Array. Known quantities of each poly A⁺ RNA were applied to eight membranes. Each membrane was hybridized to one of eight housekeeping genes. The hybridization signal produced by each sample was quantified using a Storm PhosphorImager. For each poly A⁺ RNA sample, the average relative intensity was calculated and plotted on the y-axis. Refer to Figure 2 for identification of the poly A⁺ RNAs.

Each MTE Array is prepared using CLONTECH's Poly A⁺ Premium RNA™, which provides full-length transcripts, rare transcripts, and virtually no genomic DNA. By preparing the MTE Array with poly A⁺ RNA instead of total RNA, nonspecific background is reduced, and the detection sensitivity is increased. Increased sensitivity allows you to detect lower levels of gene expression that can be confirmed by RT-PCR.

MTE™ Arrays ensure accurate expression profiling

CLONTECH ensures the most accurate expression profiles possible by normalizing the MTE Array to not one, but *eight* different housekeeping genes. The term *normalization* refers to the process of using the expression levels of housekeeping genes to determine the quantity of poly A⁺ RNA to load onto each MTE Array (1). Figure 3 shows that because the expression levels of most genes vary widely between tissues, membranes loaded with equal gram quantities of poly A⁺ RNA inaccurately reflect the expression profiles of target genes. As shown in Figure 4, CLONTECH's normalization process generates an average relative intensity for all eight housekeeping genes that varies less than 25% for all 76 poly A⁺ RNAs. Overall, normalization provides an accepted internal control so that you can compare expression levels between different experiments and draw valid conclusions. For a more detailed discussion of the normalization process, see pages 30–31.

Figure 1 demonstrates the superior quality data provided by each MTE Array. The MTE Array in Figure 1A was hybridized with CLONTECH's Human Ubiquitin cDNA Control Probe (#9806-1), a constitutively expressed housekeeping gene. Because of the normalization process, a relatively consistent hybridization signal is produced for all samples. The minor variations reflect the actual tissue-specific expression profile of ubiquitin. Figure 1B shows that the tissue-specific hybridization signals obtained using an MTE Array are highly specific. In this experiment, a probe for the tyrosine aminotransferase (TAT) gene, which is known to be expressed specifically in the liver, predominately hybridizes to adult and fetal liver.

Human MTE™ Array...continued

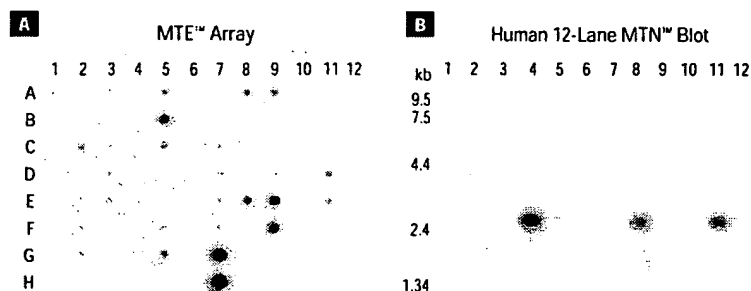


Figure 5. Expression profiles of the human lactoferrin gene. Each membrane was hybridized with a lactoferrin cDNA probe overnight; hybridization was performed as in Figure 1. **Panel A.** MTE™ Array. **Panel B.** Human 12-Lane MTN™ Blot (#7780-1). Lane 1: brain. Lane 2: heart. Lane 3: skeletal muscle. Lane 4: colon. Lane 5: thymus. Lane 6: spleen. Lane 7: kidney. Lane 8: liver. Lane 9: small intestine. Lane 10: placenta. Lane 11: lung. Lane 12: peripheral blood leukocyte. Note that on the 12-Lane MTN Blot, colon poly A⁺ RNA includes the mucosal lining, whereas on the MTE Array, it includes only ascending, transverse, and descending colon. This accounts for the slightly different expression profiles.

Finally, the MTE Array can be used to uncover a complex gene expression profile as shown in Figure 5A. Expression profiles allow you to speculate on the potential function of your gene and to choose tissues from which to clone a full-length cDNA for further analysis. For the most accurate quantification of gene expression, we recommend using the Storm PhosphorImager from Molecular Dynamics.

MTE™ Arrays and MTN™ Blots—complementary techniques

The Human MTE Array allows you to screen for the presence and relative abundance of a gene transcript in a broad spectrum of fetal and adult tissues. Because each poly A⁺ RNA sample is confined to a small 1-mm dot, the sensitivity of detection with an MTE Array is superior to what can be achieved using a Northern blot. However, to distinguish between different-sized transcripts, we recommend using CLONTECH's Multiple Tissue Northern (MTN™) Blots. Figure 5 demonstrates that because many of the same Poly A⁺ Premium RNAs are found on both the MTE Array and MTN Blots, you can obtain complementary data. The MTE Array in Figure 5A reveals the expression profile and relative abundance of

human lactoferrin, while the Human 12-Lane Multiple Tissue Northern (MTN™) Blot (#7780-1; 2) in Figure 5B reveals the expression profile and size of lactoferrin transcripts. For a complete listing of available MTN Blots, see our catalog or visit our web site at mtn.clontech.com.

Complete hybridization system

The MTE Array comes as a complete hybridization system that ensures accuracy and consistency. Several negative controls, which should not hybridize to a human gene-specific probe, are included on each MTE Array (Figure 2; column 12). Hybridization of your probe to *E. coli* rRNA or DNA; synthetic poly r(A); yeast tRNA or total RNA; or C₀t-1 DNA reveals undesirable sequences in the probe which may mask gene-specific hybridization.

Each array is shipped with CLONTECH's ExpressHyb™ Hybridization Solution, a novel low-viscosity hybridization solution that not only reduces nonspecific background, but also reduces hybridization time (3). The Human Ubiquitin cDNA Control Probe is provided as a positive control for your labeling and hybridization procedures.

Product	Size	Cat. #
Human Multiple Tissue Expression (MTE) Array	each	7775-1

Components

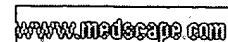
- Human MTE™ Array
- Human Ubiquitin cDNA Control Probe
- ExpressHyb™ Hybridization Solution
- Complete User Manual (PT3307-1)

Related Products

- Mouse RNA Master Blot™ (#7771-1)
- Multiple Tissue Northern (MTN™) Blots (many)
- Multiple Tissue cDNA (MTC™) Panels (many)
- ExpressHyb™ Hybridization Solution (#8015-1, -2, -3)
- Human cDNA Control Probes
 - 23-kDa Highly Basic Protein (#9802-1)
 - α-Tubulin (#9803-1)
 - β-Actin (#9800-1)
 - G3PDH (#9805-1)
 - HPRT (#9807-1)
 - Phospholipase A2 (#9804-1)
 - Ribosomal Protein S9 (#9801-1)
 - Ubiquitin (#9806-1)
- CHROMA SPIN™ Columns (many)

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Polyclonal Anti-PSA Is More Sensitive but Less Specific Than Monoclonal Anti-PSA: Implications for Diagnostic Pathology

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Abstract and Introduction

Abstract

Prostate-specific antigen (PSA) production by nonprostatic tissues has been reported, casting doubts on its specificity. The immunohistochemical relative specificity and sensitivity of PSA expression using monoclonal and polyclonal anti-PSA was analyzed on 60 prostate carcinomas, 40 normal seminal vesicles, and 310 nonprostatic tumors. All nonprostatic tumors proved negative with both antibodies. However, 13 (32%) seminal vesicles showed immunoreactivity with polyclonal anti-PSA, but none showed immunoreactivity with the monoclonal antibody. The sensitivity of the 2 antibodies for prostate cancer varied with tumor grade. In Gleason pattern 3, both antibodies showed diffuse immunostaining in all cases. In Gleason pattern 5, polyclonal anti-PSA showed diffuse (>95%) tumor cell positivity in 18 cases (90%), while with the monoclonal antibody, 7 cases (35%) showed only focal (<10%) tumor cell immunoreactivity. Thus, monoclonal anti-PSA seems to be useful in small gland proliferations in which the differential diagnosis includes seminal vesicle, while for poorly differentiated neoplasms, polyclonal anti-PSA is considered superior. Sections of high-grade prostate cancer should be included as positive controls for PSA immunostaining.

Introduction

Immunohistochemical analysis using antibodies directed against prostate-specific antigen (PSA), a secretory product of prostatic epithelium, is widely used to establish the prostatic origin of metastatic adenocarcinoma in diagnostic histopathology practice. This has important clinical relevance, as the confirmation of prostatic origin would result in the patient being administered specific treatment, which is hormonal manipulation in most instances. PSA also is useful to distinguish seminal vesicle epithelium from prostatic cancer in needle biopsy specimens, a differential diagnosis of small acinar proliferation that is complicated further by the fact that pigment resembling that of the seminal vesicle may be seen in benign and malignant prostatic epithelium.^[1] PSA also is used to distinguish prostatic adenocarcinomas from other nonprostatic small acinar proliferations or normal histoanatomic variants such as Cowper glands, nephrogenic adenoma, and hyperplastic mesonephric remnants, as well as adenocarcinomas of rectum, urethra, and urinary bladder, which may have very similar histologic features and manifest in a pelvic location.

The use of PSA immunohistochemical analysis in the aforementioned clinical settings is based on the premise that PSA is specific for prostatic origin.^[2] However, there have been several individual case reports and small series documenting PSA immunoreactivity in a variety of nonprostatic tumors, including salivary gland neoplasms, carcinoma of the breast, urothelial (transitional cell) carcinoma, adenocarcinoma of the urinary bladder, colonic adenocarcinoma, and lung carcinoma.^[3-7] PSA immunoreactivity also has been reported in benign seminal vesicle epithelium.^[8,9]

PSA immunoreactivity can be demonstrated using polyclonal and monoclonal antibodies that may differ in their sensitivity and specificity for PSA. Traditionally, polyclonal anti-PSA is used widely in the United States, while several major centers in the United Kingdom use the monoclonal antibody. However, this choice of antibody type does not seem to be based on any detailed comparison of the 2 antibodies. Previous studies evaluating PSA immunohistochemical analysis generally have focused on either the sensitivity or the specificity of immunostaining, but not both together. However, a very specific technique may lack sensitivity, while improvements in sensitivity may be at the expense of specificity.

The aim of the present study was to systematically analyze the immunohistochemical specificity and sensitivity of PSA using both monoclonal and polyclonal anti-PSA applied with a contemporary antigen-retrieval method on a large series of cases spanning the range of prostatic carcinoma differentiation and in a diverse group of nonprostatic tissues, including those with reported cross-reactivity for PSA and tumors likely to be in the differential diagnosis of prostate cancer such as carcinoid tumors and urothelial carcinoma.

Materials and Methods

Routinely formalin-fixed and paraffin-embedded tissue sections from 60 prostate carcinomas, 40 benign seminal vesicles from radical prostatectomy specimens, and 310 nonprostatic tumors were obtained retrospectively from the histology files of the University Hospital of Wales, Cardiff; Emory University Hospital, Atlanta, GA; and the University of Texas M.D. Anderson Cancer Center, Houston.

Cases of prostate carcinoma were chosen such that a range of Gleason scores most likely to result in clinically significant prostate cancer were represented: 20 each with Gleason score 6 (3 + 3) and Gleason score 7 to 8 (3 + 4, 4 + 3, or 4 + 4) from radical prostatectomy specimens and 20 with Gleason score 10 from transurethral resections of the prostate. One representative section was selected from each individual case.

The 310 nonprostatic tumors included 40 breast adenocarcinomas (grade 1, 10; grade 2, 20; and grade 3, 10; using the Elston modification of the Bloom and Richardson grading system), 40 lung adenocarcinomas (20 each, well- and poorly differentiated), 40 colonic adenocarcinomas (20 each from the right side and the left side of the colon), 40 renal cell carcinomas (20 each, Fuhrman grade 1-2 and grade 3-4), 40 urothelial carcinomas (20 each, World Health Organization grade 1 and grade 3), 30 glandular lesions of the urinary bladder (urothelial carcinomas with glandular differentiation, 20; primary bladder adenocarcinoma, 10), 40 carcinoid tumors (20 each, lung and gastrointestinal), and 40 salivary gland tumors (20 each, pleomorphic salivary adenoma and salivary gland carcinoma types).

The H&E-stained slides of all cases were reviewed to confirm the diagnosis and grade and to ensure that representative tissue (well-fixed and nonnecrotic) was selected for immunostaining.

Tissue sections from each selected paraffin block were cut on frosted slides and immunostained using monoclonal anti-PSA (clone ER/PR8, DAKO, Ely, England; dilution of 1:160 applied for 45 minutes at room temperature) and polyclonal anti-PSA (catalog number, A0562, DAKO; dilution of 1:12,800 applied for 45 minutes at room temperature) with appropriate positive and negative controls. All cases were immunostained with both antibodies after microwave pretreatment (20 minutes in a 10-mmol/L concentration of EDTA, pH 7.0). The detection system used was the Vector Elite Universal system (Vector Laboratories, Burlingame, CA) with diaminobenzidine (Biogenex, San Ramon, CA) as the chromogen.

Immunohistochemical staining was assessed independently by 2 pathologists (M.V. and M.M.). The percentage of relevant cells staining positive and the intensity of staining (weak or strong) was recorded for each case. In prostate cancers with Gleason score 7, scoring was based only on the areas of pattern 4. Differences in scoring were resolved by subsequent simultaneous review and discussion with a third pathologist (B.J.) until consensus was reached.

Results

The immunostaining patterns of prostate adenocarcinoma using monoclonal and polyclonal anti-PSA are summarized in [Table 1](#).

In Gleason pattern 3 prostate cancer, immunostaining with both antibodies was similar with diffuse PSA expression (>95% of tumor cells positive) in all cases (Figure 1). Strong immunostaining in more than 75% of cells was present

with each antibody type in 19 cases (95%). In Gleason pattern 4 tumor, polyclonal antibody was more sensitive, with diffuse immunoreactivity (>95% cells positive) in 19 (95%) of 20 cases compared with 14 (70%) with monoclonal anti-PSA. Strong immunostaining in more than 75% of cells was observed in 18 (90%) of 20 cases with the polyclonal antibody and in 11 (55%) with the monoclonal antibody.

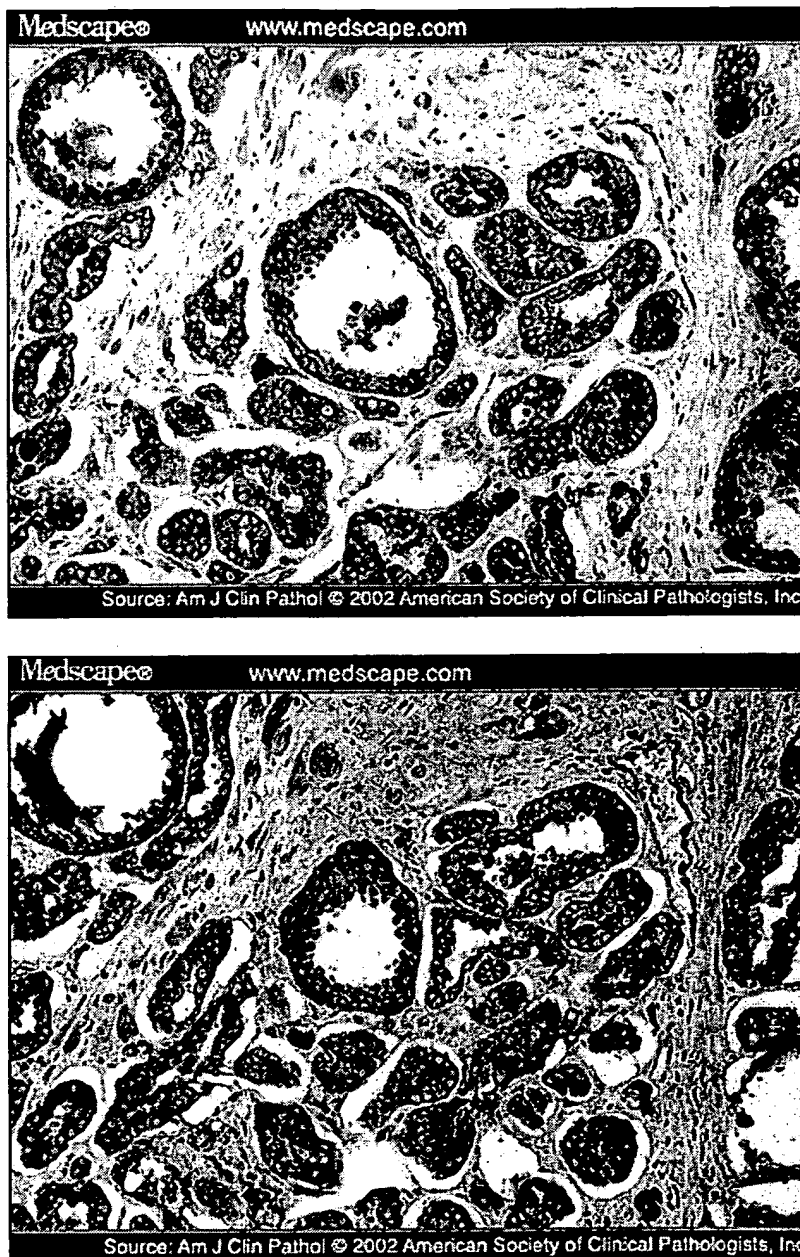


Figure 1. Prostate-specific antigen (PSA) immunohistochemical analysis in Gleason pattern 3 prostate cancer with monoclonal anti-PSA (A, avidin-biotin complex-diaminobenzidine [ABC-DAB], x20) and polyclonal anti-PSA (B, ABC-DAB, x20) showing diffuse immunoreactivity with both antibodies. Note that immunoreactivity is slightly more intense with the polyclonal antibody.

The difference between polyclonal and monoclonal anti-PSA was most marked in Gleason pattern 5 tumor (Figure 2). Monoclonal antibody was not sensitive in these tumors, with only 2 (10%) of 20 cases showing diffuse positivity and immunoreactivity in fewer than 10% of cells in 7 (35%) cases. Although none of the cases were entirely negative, 6 (30%) of 20 cases had chips from transurethral resections of the prostate with tumor cells totally negative for PSA (1-11 [median, 2.5] of 5-29 [median, 8.5] chips with tumor). Of 20 cases, 15 (75%) had chips with fewer than 5% of the tumor cells positive (8%-96% of chips; median, 50%). Polyclonal anti-PSA was more sensitive, with 18 (90%) of 20 Gleason pattern 5 tumors showing diffuse positivity and none of the cases showing fewer than

25% cells positive. None of the chips showed fewer than 25% positive cells using the polyclonal antibody. Diffuse staining was observed even in chips with cautery artifact.

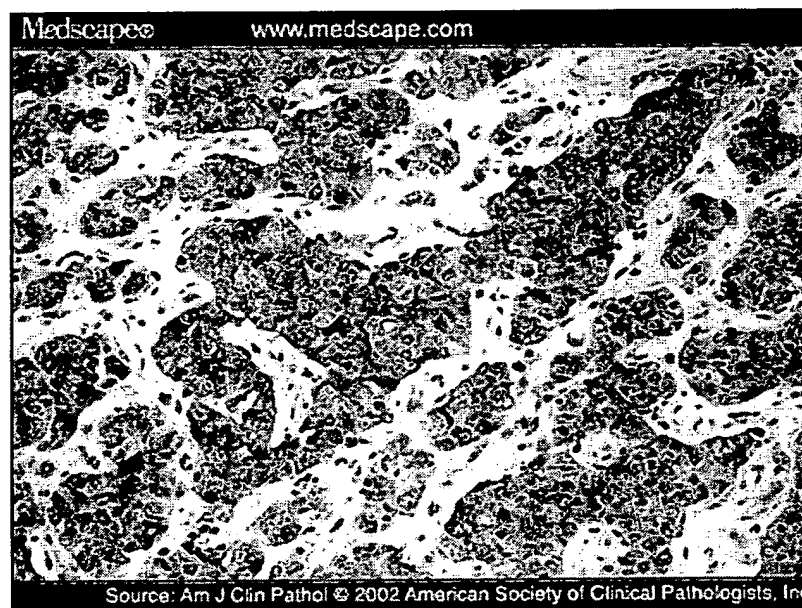


Figure 2. Prostate-specific antigen (PSA) immunohistochemical analysis in Gleason pattern 5 showing no immunostaining with monoclonal anti-PSA (A, avidin-biotin complex-diaminobenzidine [ABC-DAB], x20) but diffuse, moderately intense immunoreactivity with polyclonal anti-PSA (B, ABC-DAB, x20).

All 310 nonprostatic tumors studied were negative with both monoclonal and polyclonal anti-PSA. However, with polyclonal anti-PSA, diffuse strong immunoreactivity was present along the luminal aspect of nonneoplastic salivary ducts in 27 (90%) of 30 salivary gland tumors in which nonneoplastic salivary glandular tissue was represented. No immunoreactivity was observed in these nonneoplastic ducts with monoclonal anti-PSA. Of 40 sections of seminal vesicle, 13 (32%) showed cytoplasmic immunoreactivity with polyclonal anti-PSA (1%-40% of cells positive; mean, 15.5%). The intensity of immunoreactivity was strong in 8 (62%) of these cases (3%-15% of cells; mean, 8.5%). All sections of seminal vesicle were negative with monoclonal anti-PSA (Figure 3).

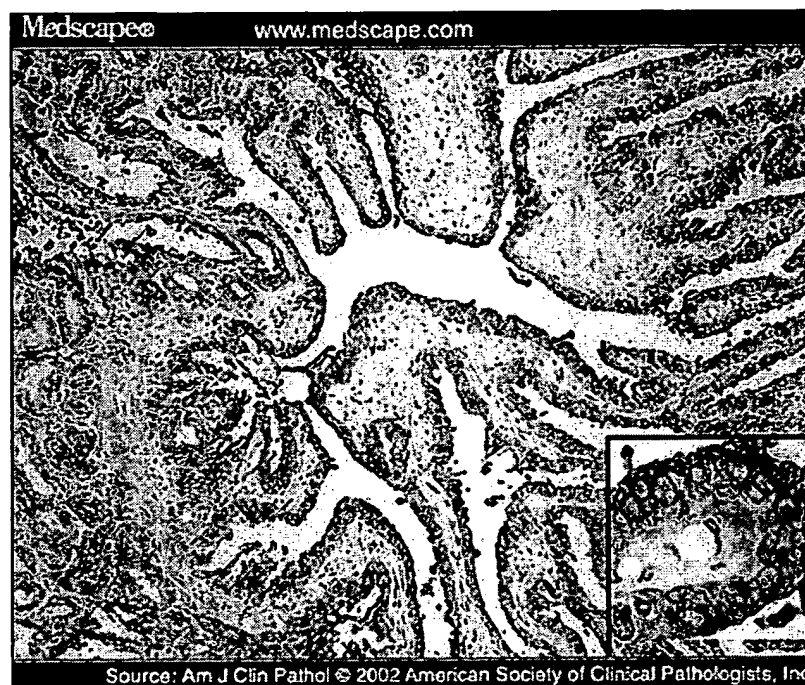
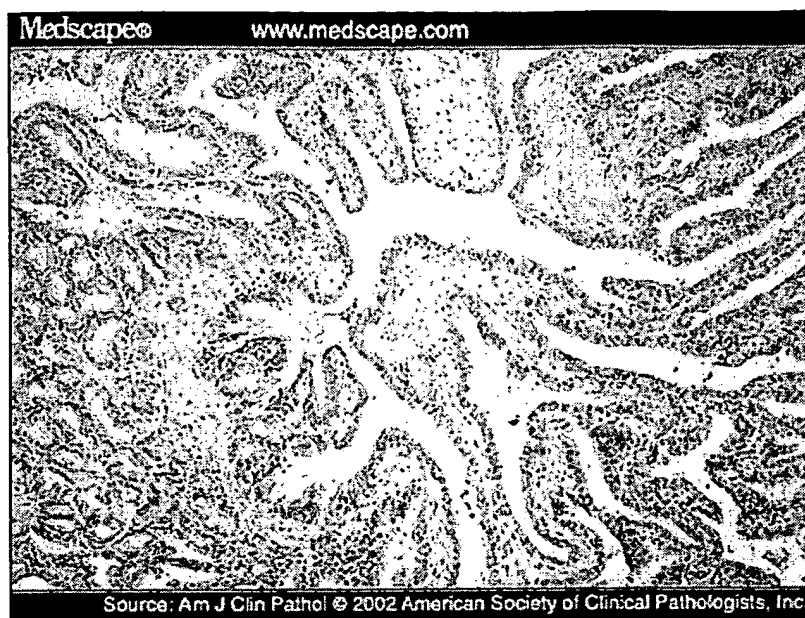


Figure 3. Seminal vesicle showing no immunostaining with monoclonal anti-prostate-specific antigen (anti-PSA) (A, avidin-biotin complex-diaminobenzidine [ABC-DAB], $\times 10$) but intense immunoreactivity with polyclonal anti-PSA (B, ABC-DAB, $\times 10$). Inset shows moderate to strong cytoplasmic polyclonal anti-PSA immunostaining in seminal vesicle epithelium (ABC-DAB, $\times 40$).

Discussion

Conflicting views have been expressed about the tissue specificity of PSA. While PSA generally has been considered specific for prostatic origin, more recently Levesque et al^[5] described PSA as "a relatively ubiquitous glycoprotein...perhaps a growth factor." In our experience, PSA as detected by immunohistochemical analysis is very tissue-specific, as we did not find PSA immunoreactivity in any of the 310 nonprostatic tumors studied using monoclonal and polyclonal anti-PSA with heat-induced epitope retrieval (HIER). In view of the molecular biologic evidence of PSA production by nonprostatic tissues,^[10,11] it is likely that PSA is produced by tissues other than the prostate gland but at levels of expression too low to be detected by even the highly sensitive immunohistochemical

techniques used at present. Our results are different from those of previous studies that showed immunohistochemical expression of PSA in some nonprostatic tumors.^[3,4,6,7] This is probably related to technical differences such as the source of the polyclonal antibody used. It has been suggested that at least 1 batch of polyclonal anti-PSA that had been used in some of the studies may have cross-reacted with cytokeratins.^[12]

While we did not find PSA expression in any of the nonprostatic tumors studied, notably urothelial carcinoma, adenocarcinoma of the urinary bladder, and carcinoid tumors, we did observe PSA positivity in the ductal epithelium of nonneoplastic salivary glands and in almost one third of the seminal vesicles with polyclonal but not monoclonal anti-PSA. These findings are similar to those reported in previous studies.^[3,8,9] PSA immunoreactivity in benign salivary ductal epithelium is interesting but of little clinical significance. In contrast, distinction of prostatic adenocarcinoma from glands of the seminal vesicle is important in 2 different settings. In prostate needle biopsy specimens, the glands of the seminal vesicle may mimic prostatic adenocarcinoma owing to the small acinar histologic features and nuclear atypia resulting in a possible false-positive diagnosis of cancer. In radical prostatectomies, rarely the peripheral budding and outpouchings of the seminal vesicle epithelium results in small acinar morphologic features with an infiltrative character such that carcinomatous involvement of the seminal vesicle becomes a serious consideration. Immunohistochemical analysis may be necessary to resolve this problem that was emphasized in the third series of the Armed Forces Institute of Pathology fascicle on the prostate and the seminal vesicle.^[13] However, to avoid misdiagnosing or overstaging prostate cancer, it is important to recognize that PSA expression may be detected in the seminal vesicle epithelium when polyclonal antibody is used. Possible explanations for PSA expression in the seminal vesicle include low-level aberrant expression demonstrated only by the more sensitive polyclonal antibody, nonspecific cross-reaction of polyclonal anti-PSA with some other antigen in seminal vesicle epithelium, or retrograde movement of PSA from prostatic ducts into the seminal vesicle.

Like previous studies, we found that PSA expression varies with the grade of prostate cancer.^[14-16] The percentage of positive cells and intensity of immunostaining decreased progressively with increasing tumor grade, especially with monoclonal anti-PSA. This may reflect heterogeneity in poorly differentiated cancer and may explain at least in part the relatively low serum PSA levels observed in some cases of poorly differentiated prostate cancer.

Polyclonal anti-PSA generally was substantially more sensitive than the monoclonal antibody in the diagnosis of prostate cancer. In Gleason pattern 3 tumor, both antibodies were very sensitive with diffuse immunoreactivity. However, the intensity of immunostaining was greater with the polyclonal antibody. The difference between the 2 antibodies was most marked in the high-grade tumors. With the monoclonal antibody, in Gleason pattern 5 tumor, diffuse staining (>95% cells positive) was seen only in 10% of cases (2/20), while fewer than 10% of cells were positive in 35% of cases (7/20). Although none of the cases were entirely immunonegative, chips with tumor totally negative for PSA were present in more than one third of cases and could be a potential source of false-negative immunohistochemical results. In contrast, polyclonal anti-PSA was highly sensitive even in Gleason pattern 5 tumors, with diffuse staining apparent in 90% of cases (18/20). Thus, differences in sensitivity of PSA immunostaining techniques may become apparent only when poorly differentiated prostate cancers are studied. Hence, we recommend that sections of high-grade (Gleason 5 + 5 = 10) prostate carcinoma also should be included as positive controls in PSA immunohistochemical analysis.

The sensitivity of PSA immunohistochemical analysis in the present study using polyclonal antibody was substantially higher than that reported in the literature (Table 2). Of the 95 cases of poorly differentiated prostate carcinoma in the 4 studies that used polyclonal anti-PSA, 7 (7%) totally lacked PSA immunostaining. In the present study, using polyclonal anti-PSA, more than 25% of tumor cells were positive in all poorly differentiated prostate cancers studied. The higher sensitivity may be related to the use of HIER in contrast with the older studies that predated the advent of this powerful technique.^[19]

The high sensitivity for prostate adenocarcinoma observed in our study using polyclonal anti-PSA with microwave HIER might be clinically significant. It has been generally accepted that while PSA positivity in a poorly differentiated carcinoma establishes prostatic origin, lack of PSA immunostaining does not rule out prostate carcinoma, as PSA expression is reduced in poorly differentiated prostate cancer.^[12] However, according to the present study, negative immunostaining in a poorly differentiated carcinoma using polyclonal anti-PSA with HIER would make a prostatic origin very unlikely.

PSA immunohistochemical analysis is confirmed to be highly specific and sensitive for establishing the prostatic origin of a tumor. In small gland proliferations in which the differential diagnosis includes the seminal vesicle, the use of monoclonal anti-PSA is recommended in view of false-positive staining of seminal vesicle epithelium with the polyclonal antibody. In metastatic prostate cancer, which is most often Gleason pattern 4 or 5 and frequently present in limited diagnostic material, polyclonal anti-PSA is considered superior to the monoclonal antibody. We

recommend that sections of poorly differentiated prostate cancer should be included as positive controls, as these would provide a more stringent check of the sensitivity of PSA immunohistochemical analysis.

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Tables

Table 1. Distribution of Prostate-Specific Antigen Immunoreactivity in Prostate Cancer, According to the Grade of the Tumor and the Type of Antibody Used*

Gleason Pattern	Antibody Type	Percentage of Cells Positive						
		0	<5	5-9	10-49	50-74	75-95	>95
3 (n = 20)	Monoclonal	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	20 (100)
	Polyclonal	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	20 (100)
4 (n = 20)	Monoclonal	0 (0)	0 (0)	0 (0)	0 (0)	2 (10)	4 (20)	14 (70)
	Polyclonal	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (5)	19 (95)
5 (n = 20)	Monoclonal	0 (0)	2 (10)	5 (25)	6 (30)	2 (10)	3 (15)	2 (10)
	Polyclonal	0 (0)	0 (0)	0 (0)	1 (5)	0 (0)	1 (5)	18 (90)

* Data are given as number (percentage) of cases.

Table 2. Prostate-Specific Antigen Immunoreactivity in Poorly Differentiated Prostate Cancer: A Review of the Literature*

Reference and Year	No. of Cases	Antibody	Pretreatment	Percentage of Cells Positive			
				0	<5	<25	<50
Stein et al, ^[14] 1982	7	Polyclonal	None	29	NA	NA	NA
Ellis et al, ^[15] 1984	20	Polyclonal	None	5	25	25	25
Svanholm, ^[16] 1986	42	Polyclonal	None	5	NA	NA	NA
Keillor and Aterman, ^[17] 1987	20	NA	Trypsin	10	NA	NA	NA
Cho and Epstein, ^[18] 1987	26	Polyclonal	None	8	20	48	48
Present study	20	Polyclonal	Microwave	0	0	0	5

NA, data not available.

* Data are given as percentage of cases.

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